CHARACTERIZATION OF CATION/H^+ ANTIPORTERS FROM *ARABIDOPSIS* AND ZEBRAFISH

A Dissertation

by

HUI MEI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2009

Major Subject: Molecular and Environmental Plant Sciences

CHARACTERIZATION OF CATION/H^+ ANTIPORTERS FROM *ARABIDOPSIS* AND ZEBRAFISH

A Dissertation

by

HUI MEI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Co-Chairs of Committee,	Kendal Hirschi
	Jean Gould
Committee Members,	Carol Loopstra
	Daniel Lineberger
Chair of Interdisciplinary Faculty,	Jean Gould

May 2009

Major Subject: Molecular and Environmental Plant Sciences

ABSTRACT

Characterizations of Cation/H^+ Antiporters from *Arabidopsis* and Zebrafish.

(May 2009)

Hui Mei, B.S., YunNan University

Co-Chairs of Advisory Committee: Dr. Kendal Hirschi
Dr. Jean Gould

To maintain optimal cytosolic Ca^{2+} concentrations, cells employ three distinct strategies: 1) tightly regulated influx of Ca^{2+}; 2) efficient efflux of Ca^{2+} from the cell; 3) sequestration of Ca^{2+} in organelles. Ca^{2+}efflux and influx are mediated by diverse transporter systems, such as pumps, channels and antiporters. Vacuolar localized Ca^{2+}/H^+ exchangers such as *Arabidopsis thaliana* cation exchanger 1 (CAX1) play important roles in Ca^{2+} homeostasis. When expressed in yeast, CAX1 is regulated via an N-terminal autoinhibitory domain. I compared and contrasted the properties of N-terminal CAX1 variants in plants and yeast expression systems to determine if autoinhibition of CAX1 is occurring in *planta*. Although several plant transporters appear to contain N-terminal autoinhibitory domains, my work is the first to document clearly N-terminal-dependent regulation of a Ca^{2+} transporter in transgenic plants.

In my second study, I characterized another *Arabidopsis* CAX, *CAX4. CAX4* is expressed in the root apex and lateral root primordia and expression increased when Ni^{2+} or Mn^{2+} levels were elevated or Ca^{2+} was depleted. Transgenic plants expressing increased levels of *CAX4* displayed symptoms consistent with increased sequestration of Ca^{2+} and Cd^{2+} into the vacuole. When CAX4 was highly expressed in an *Arabidopsis*

cax1 mutant line with weak vacuolar Ca^{2+}/H^+ antiport activity, a 29% increase in Ca^{2+}/H^+ antiport was measured. A *cax4* loss-of-function mutant and *CAX4* RNA interference lines displayed altered root growth in response to Cd^{2+}, Mn^{2+}, and auxin. The *DR5::GUS* auxin reporter detected reduced auxin responses in the *cax4* lines. My results indicate that CAX4 is a cation/H^+ antiporter that plays an important function in root growth under heavy metal stress conditions.

My third study is to characterize a zebrafish CAX. In this study, I have initiated characterization of a zebrafish CAX by initially expressing the open reading frame in yeast. Zebrafish Cax1 was localized on the yeast endomembranes and displayed Ca^{2+}/H^+ activity. In zebrafish embryos, Cax1 was specifically expressed in neural crest cells and morpholino knockdowns of cax1 caused defects in neural crest development as measured by alterations in pigmentation, defects in jaw development and reduction in expression of the neural crest marker, pax7. Collectively, our findings provide a previously unexpected role of CAX transporters in animal growth and development.

ACKNOWLEDGEMENTS

In the first place I would like to express profound gratitude to my committee co-chair, Dr. Kendal Hirschi, for his invaluable support, encouragement, supervision, critique, and guidance throughout my research work. I am deeply indebted to my committee co-chair, Dr. Gould, who is always patient, ready to provide her support and continuous guidance that enabled me to complete my work successfully. Without her support, my research project would not have gone this far. I also gratefully thank my committee members, Dr. Loopstra and Dr. Lineberger, for their support, advice, knowledge, and guidance throughout the duration of my research.

I would like to thank Dr. Sunghun Park, Dr. Ning Hui Cheng, Dr. Jon Pittman, and Dr. Toshiro Shigaki, for all their tremendous help with my various experimental projects and valuable advice in scientific discussions, Dr. Bruce B. Riley and his lab members for help with the zebrafish project, and Dr. Michael Gustin and Dr. Hans-Ulrich Mösch for the help with yeast genetics.

I would like to thank my husband, Jinglan Zhang, for supporting and enouraging me to pursue this degree. Without my husband's support, I would not have finished the degree.

I wish to thank my parents, Xiaofei Mei and Mingfen Kong, for their support and love throughout my life. To them I dedicate this work.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION: REVIEW OF LITERATURE

To maintain optimal cytosolic Ca^{2+} concentrations, cells employ three distinct strategies: 1) tightly regulated influx of Ca^{2+}; 2) efficient efflux of Ca^{2+} from the cell; and 3) sequestration of Ca^{2+} in organelles. Ca^{2+}efflux and influx are mediated by diverse transporter systems, such as pumps, channels and antiporters. Yeast (*Saccharomyces cerevisiae)* are unicellular fungi that have a relatively small genome but complex internal cell structures, which are similar to those of plant cells. The availability of mutant yeast strains lacking endogenous transport systems provides an efficient tool to study Ca^{2+} transporters from higher eukaryotes upon their expression in yeast cells. The discovery, biochemical characterization, and protein designing of plant transporters have been aided by yeast systems. Of special note, yeast played an indispensable role in the study of CAX-type cation/H^+ exchangers. Therefore, yeast is a tractable model for studying the biochemical properties and physiological functions of Ca^{2+} transporters from plants.

In this review, I will emphasize the use of the yeast as a model system to study Ca^{2+} transporters. I will first review current investigations on yeast Ca^{2+} transporters and their roles in Ca^{2+} signaling. In the second part of this review, a group of cation/H^+ exchangers termed CAXs (CAtion/H^+ eXchangers) will be reviewed to highlight the application of yeast system in plant transporter research.

This dissertation follows the style of The Plant Cell.

Cytosolic Ca^{2+} homeostasis in budding yeast (*Saccharomyces cerevisiae*)

Yeast are unicellular fungi that provide a tractable model for biological studies. In terms of industrial applications, the budding yeast *Saccharomyces cerevisiae* has been used in baking and alcoholic beverage fermentations for thousands of years (Ostergaard et al., 2000). In the laboratory, tremendous progress in molecular biology, genomics, and computer technology has resulted in great advances in yeast research. *S. cerevisiae* has a relatively small genome, predicted to encode ~6200 proteins, and was the first eukaryote to have its genome completely sequenced (Goffeau et al., 1996). Other experimental advantages include ease of culture, high efficiency in transformation, complex internal cell structures (similar to plant and animal cells) and the availability of genome wide deletion mutants. A number of online databases, for example, the Saccharomyces Genome Database (http://www.yeastgenome.org/) catalogs the results from yeast studies and provides a comprehensive database of yeast related research. All these tools make yeast one of the most intensively researched eukaryotic organisms.

Many proteins and signaling pathways are conserved among eukaryotic organisms. It was clear long before the systematic sequencing of yeast genomes began that there are genes in yeast that encode proteins similar to more "complex" organisms (Botstein and Fink, 1988). The homologies are not only of sequence but also of biological function (Botstein et al., 1997). Thus, by studying yeast, we can make valuable insights into a variety of biological systems. For example, the understanding of Ca^{2+} homeostasis in yeast cells provides a platform to delineate Ca^{2+} signaling networks in other organisms.

Calcium plays an indispensable role in eukaryotic cells. Particularly, Ca^{2+} acts as an important second messenger in the transduction of external signals through the cytoplasm (Cunningham and Fink, 1994; Bonilla and Cunningham, 2002). Intracellular Ca^{2+} concentrations act as a signal to regulate a variety of physiological changes. In yeast cells, Ca^{2+} plays a role in the mitotic cycle and mating process (Anraku et al. 1991), salt and cold stresses (Batiza et al., 1996; Bonilla and Cunningham, 2003; Matsumoto et al., 2002), nutrient sensing and the cell integrity response (Ton and Rao, 2004).

In resting yeast cells, cytosolic free Ca^{2+} is actively maintained in the range of 50-200nM in the presence of environmental Ca^{2+} concentration ranging from <1μM to > 100mM (Miseta et al., 1999). In response to a variety of external stimuli, Ca^{2+} -signaling is generally initiated by releasing of Ca^{2+} from intracellular organelles. Ca^{2+} signal transduction can be divided into four components (Berridge et al., 2000; Winslow and Crabtree, 2005):

- Stimulus
- Release Ca^{2+} into cytoplasm
- Ca^{2+} functions as second messengers
- Restoration of the low cytosolic free Ca^{2+} level

Previously, several external stimuli were shown to evoke calcium signaling including mating pheromones, salt stress, glucose-1-phosphate accumulations, and depletion of Ca^{2+} from secretory organelles (Birchwood et al., 2001). Each of these signals causes various physiological responses including biochemical responses and

alterations in gene expression. Opening of Ca^{2+} channels in the plasma membrane and certain organellar membranes is the initial step of Ca^{2+} signaling. The massive influx of Ca^{2+} into the cytosol down its concentration gradient increases cytosolic Ca^{2+} levels 10- to 100-fold (Cunningham and Fink, 1994). The released Ca^{2+} binds to and activates regulatory proteins such as calmodulin, which regulates downstream events. The restoration of the cytosolic Ca^{2+} level is soon followed by channel closure and activation of Ca^{2+} pumps and antiporters to remove Ca^{2+} from the cytoplasm.

Our understanding of how the Ca^{2+} channels, pumps and antiporters work together to maintain the Ca^{2+} levels in the cytoplasm of yeast cells has been significantly increased with the recent cloning and characterization of key transporters and regulators. Under normal conditions, extracellular Ca^{2+} enters the cytosol through an as of yet unidentified channel. Meanwhile, the Cch1p-Mid1p channel on the plasma membrane opens only under specific conditions such as pheromone stimulation and depletion of secretory Ca^{2+} (Locke et al., 2000). The vacuolar channel Yvc1p releases Ca^{2+} into the cytosol under extracellular hypertonic shock (Palmer et al., 2001). To reset calcium levels, cytosolic Ca^{2+} can be pumped into both the ER and Golgi through Pmr1p and can be sequestered into the vacuole through Pmc1p and Vcx1p (see below). The expression of *Pmc1*, *Pmr1* and *Vcx1* are regulated by calcineurin, a highly conserved protein phosphatase (Cunningham and Fink, 1996; Jingsbury and Cunningham, 2000). Calcineurin is activated by Ca^{2+}/calmodulin, and calmodulin is a small Ca^{2+}-binding protein. In response to the elevation of the cytosolic Ca^{2+} level, calmodulin binds Ca^{2+} to

activate target proteins including calcineurin (Cyert, 2001). The recent progress in understanding each of the Ca^{2+} transporters is summarized in figure 1.1.

Ca^{2+} channels

Ion channels are molecular entities found in both prokaryotes and higher eukaryotes (Bonilla and Cunningham, 2002). In yeast, the plasma membrane Ca^{2+} channel, Cch1p, was identified by its homology to mammalian L-type voltage-activated Ca^{2+} channels (VGCCs) α_1-subunit (Fischer et al., 1997; Catterall, 2000). Cch1p is required for Ca^{2+} influx in mutants lacking Pmr1p which is a Golgi localized Ca^{2+} pump (see below). Cch1p activity is also stimulated by carbohydrates, lipid biosynthesis, and ER stress (Bonilla et al., 2002). A second plasma membrane protein Midp1 is also required for Ca^{2+} influx under these conditions and during mating (Iida et al., 1994, Fischer et al., 1997). Mid1p shares homology with cyclic nucleotide-gated cation channels. In the absence of Ca^{2+}, the *mid1* mutant dies in response to mating pheromone because of a defect in Ca^{2+} influx. Mid1p interacts with Cch1p (Locke et al., 2000) and is required for Cch1p activity under various conditions in vivo (Fischer et al., 1997; Paidhungat et al., 1997; Locke et al., 2001), suggesting that Mid1p and Cch1p encode subunits of a high-affinity Ca^{2+} channel and catalyze Ca^{2+} influx into yeast. The cch1Δ mid1Δ double mutant is hypersensitive to low temperatures and to high iron concentrations in the medium and both phenotypes are suppressed by high Ca^{2+} concentrations (Peiter et al., 2005). These findings suggest Cch1pMid1p-mediated Ca^{2+} influx is essential for cold stress and oxidative stress tolerance.

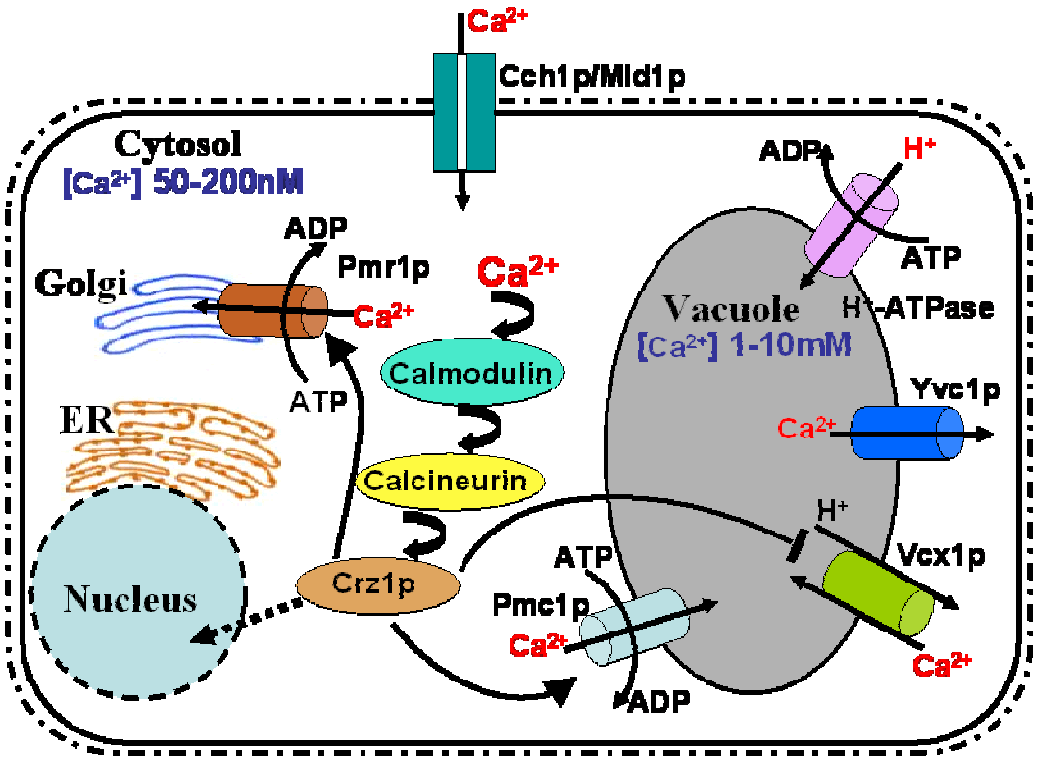


Figure 1.1 Ca^{2+} signaling pathway and transporters in *Saccharomyces cerevisiae*.

Ca^{2+} enters the cytosol via the plasma membrane channel complex Cch1p/Mid1p or the vacuolar transient receptor potential-like channel Yvc1p in response to diverse environmental cues such as ER stress, mating pheromone or hyperosmotic shock. The Ca^{2+} signaling pathway has been well characterized at the molecular level. Ca^{2+} and calmodulin activate the protein phosphatase calcineurin, which in turn activates the transcription factor Crz1p/Tcn1p, leading to its nuclear translocation and the transcription of target genes, including vacuolar Ca^{2+}-ATPase *Pmc1* and Golgi Ca^{2+}-ATPase *Pmr1*. Unlike *Pmc1* and *Pmr1*, the vacuolar H^+/Ca^{2+} exchanger Vcx1p is negatively regulated by Crz1p at post translation level. To reset the Ca^{2+} level in cytosol, Ca^{2+} is removed from the cytosol by Pmr1p, Pmc1p, and Vcx1p.

The yeast vacuolar Ca^{2+} channel Yvc1p is similar to mammalian transient receptor potential channels (TRPCs) (Palmer, et al., 2001). It has been shown by electrophysiology methods to be a cation-selective channel that can conduct Ca^{2+}, K^+, or Na^+ (Wada et al., 1987; Bertl and Slayman, 1990, 1992; Bertl et al., 1992). In vivo, Yvc1p is required for Ca^{2+} release from vacuoles under hyperosmotic shock (Denis and Cyert, 2002).

Ca^{2+} pumps

In yeast cells, ATP-driven pumps actively transport Ca^{2+} from the cytosol to extracellular space or intracellular organelles including the ER, Golgi, and vacuole. Two Ca^{2+}-ATPases have been identified, the vacuolar Ca^{2+}-ATPase Pmc1p and Ca^{2+}/Mn^{2+}-ATPase Pmr1p which is localized on Golgi.

Vacuolar membrane Ca^{2+} pump Pmc1p is similar in sequence to mammalian plasma membrane Ca^{2+} -ATPases (PMCAs; Cunningham and Fink, 1994). Cells lacking Pmc1p are unable to grow in the media containing 200mM Ca^{2+} and accumulate 20% less intracellular Ca^{2+} than wild-type cells, while deletion of the vacuolar Ca^{2+}/H^+ exchanger Vcx1p only decreases Ca^{2+} tolerance slightly (see below), suggesting that Pmc1 plays a more significant role in vacuolar Ca^{2+} sequestration in response to high extracellular Ca^{2+} level (ref?).

The Golgi-localized Ca^{2+}/Mn^{2+}-ATPase Pmr1p is important for secretory pathway functions by supplying the Golgi lumen with Ca^{2+} and Mn^{2+} for protein sorting and glycosylation. The *pmr1Δ* mutant strain is hypersensitive to excessive Mn^{2+} in the medium or removal of Ca^{2+} from the medium by the chelator BAPTA (Sorin et al., 1997;

Yadav et al., 2007). This sensitivity to the lack of Ca^{2+} in pmr1 mutants is due to the Ca^{2+} starvation in the early part of the secretory pathway and it can be suppressed by the addition of Ca^{2+} in the medium. The Mn^{2+} hypersensitivity of *pmr1Δ* mutants corresponds to the accumulation of toxic levels of cellular Mn^{2+}. The pmr1 *Δ*mutant yeast cells also display missorting and misprocessing of proteins along the secretory pathway.

Ca^{2+} exchanger

In yeast, the vacuole functions as a cellular storage compartment in Ca^{2+} signaling and detoxification of elevated extracellular Ca^{2+} concentrations. As mentioned before, The Ca^{2+} ATPase Pmc1p and Ca^{2+}/H^{+} exchanger Vcx1p mediate sequestration of Ca^{2+} into the vacuole. The Vcx1p is a high capacity, low affinity Ca^{2+}/H^{+} exchanger driven by the proton electrochemical gradient generated by the vacuolar H^{+}-ATPase (Cunningham and Fink, 1995; Miseta and Bedwell, 1999, Pittman et al., 2004). Although deletion of *VCX1* does not cause considerable Ca^{2+} sensitivity, the deletion of both *PMC1* and *VCX1* genes leads to a greater sensitivity to Ca^{2+} than the loss of either gene alone, (Pozos et al., 1996) and Vcx1p is required to rapidly restore basal cytosolic Ca^{2+} levels following a Ca^{2+} shock (Miseta et al., 1999).

Regulation of Ca^{2+} transporters

Calmodulin and calcineurin are two critical mediators in the Ca^{2+} signaling transduction pathway (Cyert, 2003). Calmodulin is a small Ca^{2+} -binding protein, and it binds Ca^{2+} via motifs called EF hands which consist of two α-helices (Babu et al., 1988). Upon binding of Ca^{2+}, the helical EF hands of calmodulin undergo a dramatic

conformational change to release free energy (Trave et al., 1995). Calcineurin is a protein phosphatase consisting of a catalytic A subunit and a Ca^{2+}-binding regulatory B subunit. When cytosolic Ca^{2+} levels are low, calcineurin is inactive. In response to environmental stress, Ca^{2+} is released to elevate cytosolic Ca^{2+} levels and calcineurin is activated by Ca^{2+}/calmodulin binding to dephosphorylate key substrates. A transcription factor Crz1p ('crazy') is the best-characterized substrate of calcineurin in yeast. Crz1p rapidly accumulates in the nucleus after being dephosphorylated by calcineurin. Crz1p has a zinc-finger domain binding to a consensus sequence termed the calcineurin-dependent response element (CDRE; Stathopoulos and Cyert, 1997). There are more than 160 genes induced by Crz1p activation and most of them contain the CDRE in their promoter regions. Vacuolar Ca^{2+}-ATPase *PMC1* is one of these genes. When yeast cells are exposed to high extracellular Ca^{2+} (Cunningham and Fink, 1994) or lack Pmr1 (Marchi et al., 1999), transcription of *PMC1* is highly induced by the calcineurin-dependent pathway. Unlike *PMC1*, vacuolar Ca^{2+}/H^{+} exchanger Vcx1p activity is apparently inhibited by calcineurin at the post translation level (Cunningham and Fink, 1996). One study has suggested that the Golgi Ca^{2+}-ATPase Pmr1p is the major Ca^{2+} pump under normal growth conditions (Marchi et al., 1999). During Ca^{2+} signaling events, intracellular Ca^{2+} levels increase, and vacuolar Ca^{2+}/H^{+} Vcx1p is activated and rapidly sequesters cytosolic Ca^{2}+ into the vacuole and attenuates the activation of Ca^{2+} signaling pathways, whereas vacuolar Ca^{2+}-ATPase Pmc1p appears to play a minimal role in the rapid sequestration of cytosolic Ca^{2+} under this condition. When the extracellular Ca^{2+} level is high, the calmodulin-calcineurin pathway is activated leading

to down-regulation of Vcx1p activity and maximizing induction of other Ca^{2+} transporters such as Pmr1p and Pmc1p. These transporters may be optimally suited to maintain growth under conditions of high environmental Ca^{2+} stress. A more recent study has concluded that Pmc1p rather than Pmr1p is the major intracellular Ca^{2+} transporter under normal conditions (Aiello et al., 2004). These conflicting roles for these transporters will require more careful experiments.

Overview of Cation/H^+ exchangers (CAXs)

The CAXs (for CAtion eXchangers) are a group of integral membrane proteins that transport Ca^{2+} and other cations out of the cytosol using the H^+ gradient generated by primary transporters. Cation/H^+ exchangers are high-capacity, low-affinity transporters that have been found in bacteria, *Dictyostelium*, fungi, plants, and lower vertebrates (Shigaki et al., 2006). For examples: Yeast Vcx1p, which has been mentioned before, is a member of the CAX family. *E. Coli* Na^+/H^+ exchanger ChaA also belongs to the CAX family (Ivey et al., 1993). *Arabidopsis thaliana* and rice have multiple CAXs. Phylogenetic analysis of the CAX genes suggests that there are three major types of CAXs: Type I CAXs are similar to *Arabidopsis thaliana* CAX1; Type II CAXs have a long-N-terminal hydrophilic region; and Type III CAXs are similar to *E. Coli* ChaA (Shigaki et al., 2006).

Type I CAXs

Type I CAXs have been physiologically characterized from several organisms and appear to be located predominantly on the vacuolar membrane (Shigaki et al., 2006; Blumwald and Poole, 1986; Kasai and Muto, 1990; Ettinger et al., 1999; Cheng et al.,

2001). The first two plant Ca^{2+}/H^{+} exchangers were identified from *Arabidopsis thaliana* by using a yeast suppression screen, and they are named CAX1 and CAX2 (Hirschi et al., 1996). CAX1 is a Ca^{2+}/H^{+} exchanger and CAX2 has a wide substrate range and transports Ca^{2+}, Cd^{2+}, and Mn^{2+} (Hirschi et al., 2000; Shigaki et al., 2003). Numerous CAX transporters have been identified from a variety of plants (Kamiya and Maeshima, 2004; Kamiya et al., 2005; Shigaki et al., 2006). To-date, there are 17 CAXs from plants in the public database. All of plant CAXs and yeast Vcx1p are Type I CAXs. The genome of *A. thaliana* appears to contain six Type I CAXs, termed CAX1 thru CAX6. The putative proteins in this group consist of approximately 400 to 800 amino acids, and the membrane topology analysis suggests that Type I CAXs contain 11 transmembrane (TM) domains with an acidic motif between TM6 and TM7 (Figure 1.2). Several functional domains have been identified from Type I CAXs including an N-terminal autoinhibitory domain (Pittman and Hirschi, 2001) and two distinct cation domains (Shigaki et al., 2001, 2003). A detailed review of N-terminal autoinhibition studies and the physiological role of CAX1 in the plant are presented in Chapter II (Mei et al., 2007). *A. thaliana* CAX3 is 77% identical (93% similar) to CAX1 but does not suppress yeast mutants defective in vacuolar Ca^{2+} transport when expressed in yeast (Shigaki et al., 2001). A 9 amino acid domain from CAX1 was identified using chimeric constructs of CAX1 and CAX3. When this 9 amino acid domain is inserted into CAX3, CAX3 can suppress yeast vacuolar Ca^{2+} transport mutants. This 9 amino acid domain is termed the calcium domain. CAX2 is a heavy metal transporter which can transport Ca^{2+}, Mn^{2+}, and Cd^{2+}. Similar chimeric constructs of CAX1 and CAX2 were made to

identify a critical Mn^{2+} domain on CAX2 (Shigaki et al., 2003). When a 10 amino acid domain from CAX1 is inserted into CAX2, the Ca^{2+} transport of CAX2 is not affected but the Mn^{2+} transport of CAX2 is completely lost. The positions of the calcium domain and the manganese domain are indicated in figure 1.2. Besides these two functional domains, a crucial His^{338} residue on CAX1 has also been identified (Shigaki et al., 2005). Mutation of His^{338} can change the CAX1 Ca^{2+} transport activity and substrate specificity. Another study with CAXs in rice has identified two highly conserved regions between TM3 and TM4 and between TM8 and TM9. These two repetitive regions have been termed repeats c-1 and c-2 (Kamiya and Maeshima, 2004). Using site-directed mutagenesis of rice CAXs, several residues involved in substrate specificity in these two repeats have been identified. For example, 5 residues (Ser^{155}, Ser^{158}, Asn^{159}, Gly^{325}, and Ser^{352}) are involved in selectivity of Mn^{2+}.

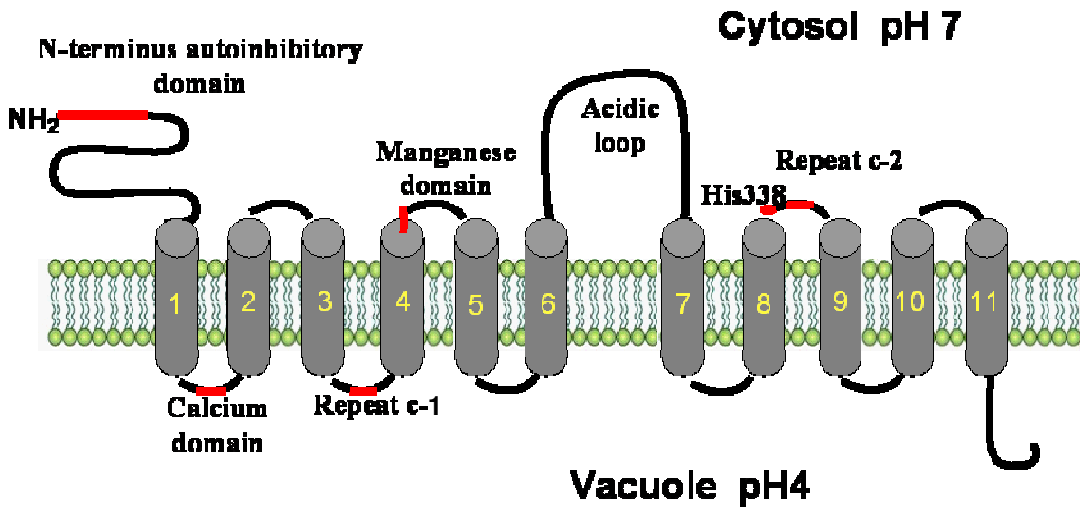


Figure 1.2 Topology model of Type I CAXs.

These detailed studies would not have been possible if the plant CAXs had not been expressed and screened in yeast. The yeast system was ideal due to its fast growth, ease of protein expression, and reproducible and highly screenable phenotypes. Moreover, the phenotypes and biochemical properties observed in yeast have been demonstrated to be well correlated with those in plants. For example, N-terminus autoinhibition discovered in yeast was later precisely reproduced in plants [42]. Transport measurements performed in plants also were well correlated with the data from yeast. Although we must keep in mind the potential pitfalls of the heterolgous system, the results from CAX studies validate the use of yeast system. In fact, in many instances, yeast is the system of choice.

Type II CAXs

Type II CAXs are present in fungi, *Dictyostelium, Xenopus,* and some lower vertebrates, but they do not appear to be present in higher vertebrates or plants (Shigaki et al., 2006). Most genomes appear to contain only one copy of this putative exchanger. A yeast open reading frame YNL321W belongs to the Type II CAXs. This group of CAXs appears to contain unusual secondary structures and consists of approximately900 to 1150 amino acids. The membrane topology analysis reveals that Type II CAXs have two additional putative TM domains at the N-terminus in comparison with Type I and Type III CAXs (Figure 1.3).

The C-terminus half of Type II CAXs is highly similar to Type I or III CAXs that contain 11 TM domains. The first TM domain of Type II CAXs is homologous to an unknown function conserved domain (DUF307) found in small membrane proteins in bacteria and fungi. The second TM domain in Type II CAXs is homologous to the β subunit proteins of Na^+, K^+-ATPase (McDonough et al., 1990; Laughery et al., 2003). This subunit may possibly play a role in its plasma membrane localization.

The characterization of this Type CAX is one of the central topics of my dissertation. As I detail in Chapter III, using zebra fish CAX1, I am attempting to discern the function of type II CAXs.

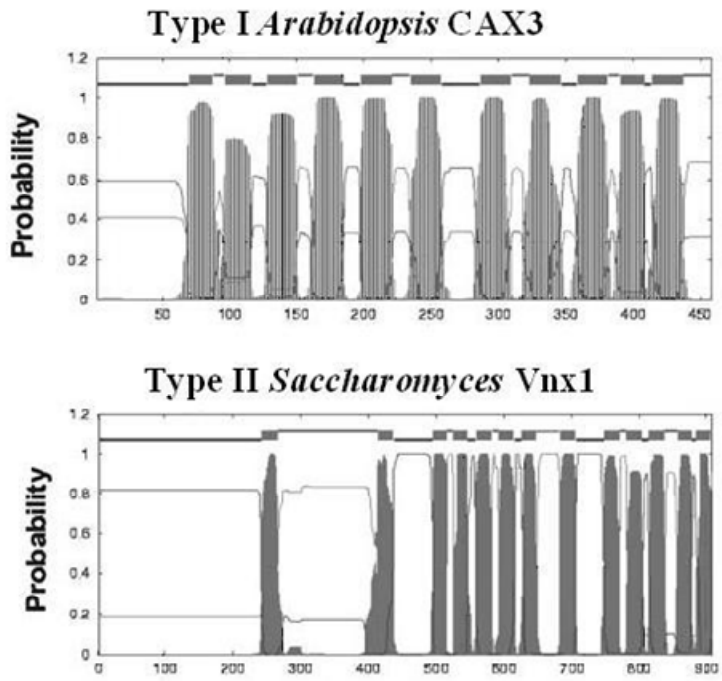


Figure 1.3 Membrane topology of Type I and Type II CAXs.

The topologic models were generated by the TMHMMprogram (Krogh et al. 2001). The top panel indicates that Type I CAXs have 11 TM domains. The bottom panel indicates that Type II CAXs have 13 TM domains (Adapted from Shigaki et al., 2006).

Type III CAXs

Type III CAXs are found only in Bacteria (Shigaki et al., 2006). Some bacterial species have both Type I and Type III CAXs. Type III CAXs contain 11 putative TM domains and approximately 400 amino acids. ChaA from *E. coli* is the only well-

characterized Type III CAX (Ivey et al., 1993; Sakuma et al., 1998). ChaA was identified by its ability to restore Na^{2+} resistance to Na^{2+}/H^{+} antiporter-deficient *E. coli* strains. Besides the Na^{2+}/H^{+} transport activity, ChaA also has a pH-independent Ca^{2+}/H^{+} activity and plays a role in calcium homeostasis at alkaline pH.

Conclusions

Transporters selectively import and export molecules and ions across membranes. Many of these membrane transport processes are well conserved from bacteria to human beings. Yeast has been used as a model in studies elucidating many cellular functions including the protein secretory pathway and membrane transport mechanisms. In the first session, I discussed: the Ca^{2+} transport in yeast by focusing on the different types of Ca^{2+} transporters and how these transporters are regulated. In the second session, I discussed the CAXs family by focusing on the characterization of *Arabidopsis* CAX1 and CAX2, identification of functional domains of CAXs, and phylogeny of CAXs.

Membrane transport is essential for almost all physiologic processes. New information, emerging from the study of model systems such as yeast and *Arabidopsis*, give us a better understanding of the nature of transporters including transporter protein structures, the substrates, their regulation, and their impact on cellular and organ functions. In the future, we can further apply this understanding to manipulate membrane transport of ions and nutrients.

CHAPTER II

IN PLANTA REGULATION OF THE *ARABIDOPSIS* Ca^{2+}/H^{+} ANTIPORTER CAX1*

Introduction

In all biological systems, the intracellular calcium (Ca^{2+}) concentrations fluctuate in response to environmental and internal cues (Sanders *et al.*, 1999; Curran *et al.*, 2000). The cytosolic Ca^{2+} levels in plant cells are tightly regulated by two opposing fluxes, the influx via Ca^{2+} channels and the efflux via Ca^{2+}-ATPases and Ca^{2+}/H^{+} antiporters (Sze *et al.*, 2000). Up to now, the regulation of these transporters has been studied almost exclusively in heterologous systems such as yeast (*Saccharomyces cerevisiae*).

Ca^{2+}/H^{+} antiporters are high-capacity, low-affinity transporters that have been physiologically characterized from a variety of plants and appear to locate predominantly on the vacuolar membrane, but also on the plasma membrane and chloroplast thylakoid membrane (Blumwald and Poole, 1986; Kasai and Muto, 1990; Ettinger *et al.*, 1999; Cheng *et al.*, 2001; Luo *et al.*, 2005; Qi *et al.*, 2005). To identify the first two plant Ca^{2+}/H^{+} antiporter genes, a yeast suppression assay was carried out (Hirschi *et al.*, 1996). Yeast strains lacking the vacuolar P-type Ca^{2+}-ATPase (*PMC1*)

---

*This chapter is reprinted with permission from "In planta regulation of the *Arabidopsis* Ca^{2+}/H^{+} antiporter CAX1" by **Mei H., Zhao J.,Pittman, J.K., Lachmansingh J.,Park S., and Hirschi, K.D.** (2007). J. Exp. Bot. © 2007

and the vacuolar Ca^{2+}/H^+ antiporter (*VCX1*) grow slowly in media containing high levels of Ca^{2+}(Cunningham and Fink, 1996). Two different *Arabidopsis thaliana* cDNAs were isolated which allowed *pmc1 vcx1* double mutant yeast stains to grow well in the media containing high levels of Ca^{2+}. These genes were termed cation exchanger (CAX) 1 and 2 (Hirschi *et al.*, 1996). Experiments utilizing vacuolar membranes from yeast cells expressing CAX1 or CAX2 demonstrate that CAX1 is a Ca^{2+}/H^+ antiporter and CAX2 is a cation/H^+ antiporter which transports Ca^{2+}, Cd^{2+}, and Mn^{2+} into the vacuole (Hirschi *et al.*, 2000; Shigaki *et al.*, 2003). Subsequently, numerous CAX transporters have been identified from various plant species (Kamiya *et al.*, 2005; Shigaki and Hirschi, 2006) and their N-terminal autoinhibitory regions defined using yeast assays (Pittman and Hirschi, 2001).

An N-terminal autoinhibitory domain controls CAX1 activity as well as other CAX transporters including VCAX1 and CAX2 (Pittman and Hirschi, 2001; Pittman *et al.*, 2002*a*, *b*, 2004). The originally identified CAX1 and CAX2 transporters are, in fact, products of partial length cDNAs and contain an N-terminal truncation which facilitates activity in yeast (now termed sCAX1 and sCAX2). *In planta*, CAX1 contains an additional 36 amino acid at the N-terminus which is not present in sCAX1. In yeast, full-length CAX1 acts as a weak vacuolar Ca^{2+}/H^+ antiporter, as transport activity is severely reduced when compared with sCAX1 (Cheng *et al.*, 2005). This autoinhibition is caused by the N-terminus physically interacting with a neighbouring N-terminal region (residues 56–62) (Pittman and Hirschi, 2001; Pittman *et al.*, 2002*a*). Further studies have identified crucial residues in these first 36 amino acids that are involved in N-terminal

autoinhibition. For example, expressing a Thr-33 to Ala variant (T33A) of CAX1 effectively suppresses Ca^{2+} sensitivity in yeast mutants in a manner indistinguishable from sCAX1 expressing cells. Mutation of a Ser residue at position 25 to Asp (S25D) also activated CAX1, suggesting this Ser may be a potential target for phosphorylation as this mutation mimics constitutive phosphorylation, while a CAX1-S25A mutation gave a CAX1 mutant that was indistinguishable from wild-type CAX1 when expressed in the Ca^{2+}-sensitive yeast cells (Pittman *et al.*, 2002*a*). However, these plant transporter activities were assayed exclusively in yeast cells and it is not known if this activity, or lack thereof, is maintained in transgenic plants expressing these constructs. To date, the only evidence to hint that autoinhibition occurs in the plant and is not purely an artefact of the yeast expression is the ability of a synthetic peptide, which corresponds to the first 36 amino acids of CAX1, to inhibit tonoplast Ca^{2+}/H^{+} transport activity in *Arabidopsis* (Pittman *et al.*, 2002*b*; Cheng *et al.*, 2003); however, a more rigorous confirmation is required.

Transgenic plants expressing CAX transporters may be a component of long-range goals to enhance plant yield and improve human nutrition (Shigaki and Hirschi, 2006). Tobacco, carrots, potatoes, and tomatoes expressing sCAX1 all contain high levels of calcium (Hirschi, 1999; Park *et al.*, 2004, 2005*a*, *b*). In some cases, when transgenic plants express sCAX1, the phenotypes are dramatic. Tobacco (*Nicotiana tabacum*) lines expressing sCAX1 exhibit Ca^{2+}-deficient symptoms such as leaf necrosis, tip burning, and hypersensitivity to ion imbalance as well as increased tonoplast Ca^{2+}/H^{+} transport activity (Hirschi, 1999). If the aim is to carefully modulate Ca^{2+}/H^{+} transport

activity in various crop plants, it is important to delineate if these phenotypes are caused by the deregulated nature of sCAX1. In addition, insights into the phenotypes associated with expression of various CAX1 variants should facilitate the rational design of transporters which maximize calcium accumulation but minimize deleterious phenotypes.

Recent technologies make it possible to measure how CAX expression modulates the sum total of all the mineral nutrients and trace elements, termed the ionome (Lahner *et al.*, 2003; Cheng *et al.*, 2005). This technology can now be harnessed as a means not only to measure phenotypes but also provide important clues about gene function. For example, yeast ionome profiles have been used to reveal the function of previously uncharacterized yeast genes (Eide *et al.*, 2005). In theory, heterologous expression and ionome measurements could be used to characterize plant transporters.

Here, N-terminal autoinhibition of CAX1 is evaluated in transgenic tobacco plants. Initially, the yeast ionome was used to compare the activity of CAX1 variants in yeast cells. CAX1 variants were then expressd in transgenic tobacco plant*s* in order to assess differences in growth and biochemical phenotypes. These results were compared with our previous findings utilizing the yeast expression assays. Collectively, the results suggest that the yeast assays provide a robust model to assess CAX autoinhibition. To our knowledge, this is the first report to demonstrate clearly the presence of N-terminal autoinhibition of transport function *in planta*. These findings also offer insights into engineering enhanced transporter activity into agriculturally important crops.

Materials and Methods

Yeast strain and plasmids

The yeast (*Saccharomyces cerevisiae*) strain K667 (*cnb1*::*LEU2 pmc1*::*TRP1 vcx1*Δ; Cunningham and Fink, 1996) was transformed and grown as described previously (Shigaki *et al.*, 2003). The *CAX1*, *sCAX1*, *CAX1-T33A*, and *CAX1-S25A* cDNA clones were subcloned into the yeast expression vector piHGpd (Nathan *et al.*, 1999) as described in previous studies (Pittman and Hirschi, 2001; Pittman *et al.*, 2002*a*).

Yeast culture conditions, sample processing, and ICP-AES analysis

Yeast culture conditions and sample processing were modified from a previous study (Eide *et al.*, 2005). Yeast cultures were inoculated in 5 ml yeast–peptone–dextrose (YPD) and 1/100 vol. of 100x mineral supplement stock (Eide *et al.*, 2005) with additional 10 mM $CaCl_2$. The cells were grown at 30 °C to stationary phase. A 2.5 ml sample of each culture was collected by vacuum filtration using isopore membrane filters (1.2 µm pore size) (Fisher Scientific, PA, USA). Cells were washed three times with 1 ml of 1 µM ethylenediaminetetraacetic acid disodium salt solution, pH 8.0, by vacuum filtration followed by three washes with 1 ml of distilled, deionized H_2O. The filters were dried at 70 °C in an oven for 48 h before inductively coupled plasma atomic emission spectroscopy (ICP-AES) analysis, performed as previously described (Lahner *et al.*, 2003).

Plant materials, transformation, and growth conditions

The generation of the *sCAX1* in the pBin19 construct has been previously described (Hirschi, 1999). The *CAX1*, *CAX1-T33A*, and *CAX1-S25A* cDNAs were subcloned into the plant expression vector pBin19 (Clontech, CA, USA), which contained the cauliflower mosaic virus (CaMV) 35S promoter fragment. The recombinant plasmids or vector controls were transformed into *Agrobacterium tumefaciens* LBA4404 (Invitrogen, CA, USA). Tobacco (*Nicotiana tabacum*) plants (cvs KY160 and KY14) were transformed along with the empty vector as a control as previously described. CAX1 and sCAX1 were expressed in KY160 (Hirschi, 1999). CAX1-T33A and CAX1-S25A were expressed in KY14 (this work).

Surface-sterilized tobacco seeds were germinated and grown as previously described (Hirschi, 1999). For ion sensitivity analysis, 14 d after plating, the seedlings were transferred to half-strength MS medium (Murashige and Skoog, 1962) containing 1% (w/v) agar supplemented with the appropriate metal salt. To make media deficient in Ca^{2+}, standard half-strength MS medium including 1% (w/v) agar was prepared, except that $CaCl_2$ was omitted. For the transport assays, tobacco seeds germinated on the standard medium were transferred to soil, and then the 4-week-old plants were transferred to a hydroponic system containing Hoagland's solution (Hoagland and Arnon, 1950).

DNA and RNA isolation: Southern and northern blot analysis

Tobacco genomic DNA was extracted from leaf tissue as previously described by Paterson *et al.* (1993). DNA (5–10 µg) was digested with *Eco*RI or *Bam*HI and separated by electrophoresis and blotted onto Hybond N^+ membrane (Amersham Biosciences, NJ, USA). Total RNA was extracted from tobacco leaves as previously described (Hirschi, 1999). RNA samples were treated with DNase to minimize any contamination of genomic DNA. The sequences of the *CAX1* gene-specific primers used in reverse transcription PCR were 5'-AAAAAATCAGACCTCCGAGTGATTCAGAA-3' and 5'-CCTTTCTCCATTGTCTCTGCTTTGGAAA-3'. Total RNA (10 µg) was separated on 1% (v/v) agarose gel containing formaldehyde, blotted onto Hybond N^+ membrane (Amersham), according to the manufacturer's instructions. PCR reactions were performed to make probes for both Southern and northern blot analysis, using a CAX1-specific primer set. The sequences of the CAX1 primers were 5'-ATGGCGGGAATCGTGACAGAGCC-3' and 5'-TTAACCCGTTTTAACTTTATTTG-3'. The membranes were prehybridized overnight at 60 °C in 7% (w/v) SDS and 0.25 M Na_2HPO_4, and then hybridized overnight at 60 °C in the same solution containing the probe labelled with ^{32}P-dCTP using a random primed labelling kit (Invitrogen, CA, USA). Membranes were washed twice for 30 min each with 2x SSC and 1% SDS at 60 °C and then washed twice again for 30 min each with 0.1x SSC and 0.5% (w/v) SDS at 60 °C. Membranes were exposed to X-ray film at –80 °C.

Preparation of membrane vesicles and Ca^{2+} transport assay

Microsomal membrane vesicles were prepared from 8-week-old hydroponically grown plant root tissue and pretreated with 100 mM $CaCl_2$ for 18 h before harvest. All membrane isolation steps were conducted at 4 °C as previously described (Hirschi, 1999). $^{45}Ca^{2+}$ uptake was measured by a filtration assay as previously described (Pittman and Hirschi, 2001).

Calcium and mineral analysis

Four-week-old tobacco plants were transferred to hydroponic growth for 4 weeks in Hoagland's solution supplemented with 2 mM $CaCl_2$. The roots were rinsed in water and harvested. The roots were dried at 70 °C for 3 d. At least 500 mg (dry weight) of root tissue was analysed individually; roots weighing less were pooled to yield the required weight as described previously (Hirschi, 1999). Total calcium and mineral contents per gram of dry mass were determined by using an inductively coupled plasma atomic emission spectrophotometer (Spectro, Kleve, Germany) as previously described (Lahner *et al.*, 2003).

Results

CAX1 expression and the yeast ionome

I was interested in assessing the impact of expressing CAX variants on the yeast ionome. To summarize previous results, the CAX1-T33A variant made CAX1 constitutively active comparable with sCAX1 while a CAX1-S25A variant was no more active than CAX1 (Pittman *et al.*, 2002*a*). In this study, these variants, the empty vector control, CAX1, and sCAX1 were expressed in the K667 (*vcx1 pmc1 cnb1*) mutant yeast

strain, which is deficient in vacuolar Ca^{2+} transporters (Cunningham and Fink, 1996), and the cells were grown in liquid media supplemented with several elements to levels sufficient to facilitate detection in cell extracts by ICP-AES. As shown in Figure 2.1A, sCAX1-expressing yeast cells accumulated Ca^{2+} approximately 4-fold compared with the vector control cells. The accumulation of calcium in the CAX1-T33A-expressing yeast cells was similar to that of sCAX1-expressing cells. Meanwhile CAX1- and CAX1-S25A-expressing yeast cells contained levels of calcium similar to the vector control cells (Figure 2.1A). In addition to the increased calcium accumulation, nickel, zinc, iron, and magnesium levels were also increased in sCAX1- and CAX1-T33A-expressing yeast cells compared with the vector control cells (Figure 2.1B). Again, these increases in other metals were not seen in the CAX1- and CAX1-S25A-expressing yeast cells. Other metal levels such as sodium, manganese, potassium, phosphorus, and copper were similar among all the yeast cells tested (Figure 2.1C).

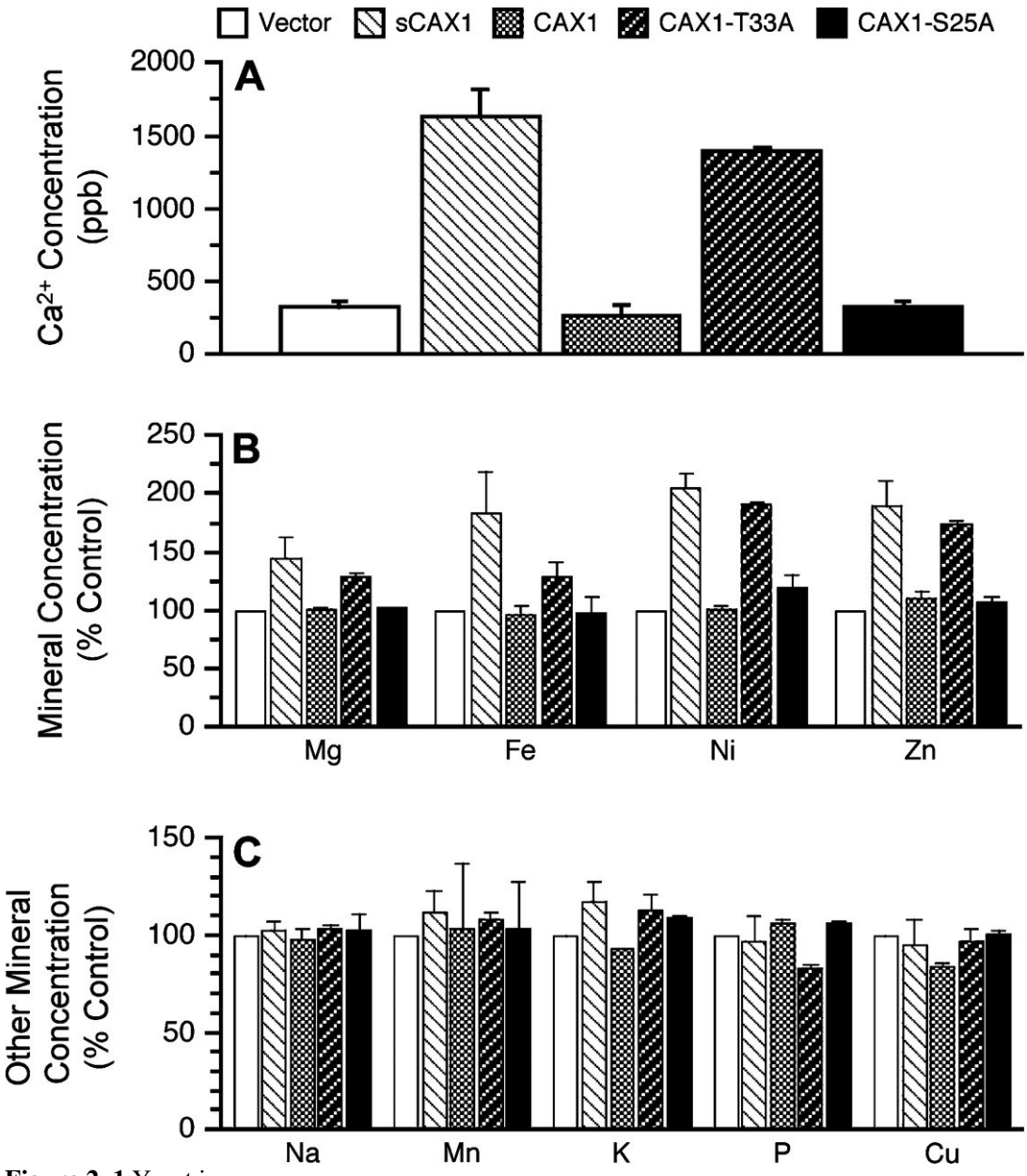


Figure 2. 1 Yeast ionome.

Concentrations of minerals in vector-, sCAX1-, CAX1-, CAX1-T33A-, and CAX1-S25A-expressing yeast cells grown in yeast-peptone-dextrose (YPD) medium with mineral supplements and additional 10 mM $CaCl_2$. (A) Total calcium concentration. (B) Total magnesium, iron, nickel, and zinc concentrations. (C) Other mineral concentrations. All the mineral concentrations were determined by ICP-AES. In (B) and (C), values were normalized to concentrations of empty vector control-containing yeast cells. Data represent the means (±SD) from three independent analyses.

CAX variants expressed in transgenic tobacco

To gain insights into the N-terminal autoinhibition of CAX1 *in planta*, empty vector controls, CAX1, sCAX1, CAX1-T33A, and CAX1-S25A were expressed in tobacco plants. It has previously been demonstrated that tobacco is an excellent plant model for assessing Ca^{2+}/H^{+} antiport function because the morphological phenotypes which result from increased vacuolar Ca^{2+} loading, such as tip burning, are easily visualized (Hirschi, 1999; Shigaki *et al.*, 2002). By contrast, the morphological differences when sCAX1 is expressed in *Arabidopsis* plants are quite subtle (Cheng *et al.*, 2003). The tobacco cultivar KY160 line expressing 35S::sCAX1 was obtained from previous studies (Hirschi, 1999). In this study, 35S::CAX1 was expressed in tobacco cultivar KY160 and 35S::CAX1-T33A and 35S::CAX1-S25A were expressed in the tobacco cultivar KY14. Initially, it was determined that KY160 and KY14 were indistinguishable in the growth assays used here except for the difference in leaf colour and morphology (data not shown). Thus, the impact of the CAX1 variants could easily be compared without regard to the differences between KY160 and KY14. As mentioned previously, tobacco lines expressing sCAX1 were previously characterized (Hirschi, 1999). This sCAX1 expressing line is representative of the multiple independent transgenic lines previously characterized. Here, at least 10 different transgenic tobacco lines expressing CAX1, CAX1-T33A or CAX1-S25A were obtained. Southern analysis was performed and it was determined that all transgenic tobacco lines analysed contained multiple insertions (data not shown). Expression of CAX1, CAX1-T33A, and CAX1-S25A was initially measured by reverse transcription PCR (data not shown). Two

independent transgenic lines expressing CAX1, CAX1-T33A, and CAX1-S25A with comparable expression levels to sCAX1 were confirmed by northern blot analysis (Figure 2.2; lines 1 and 2). Together, these observations suggested that the expression

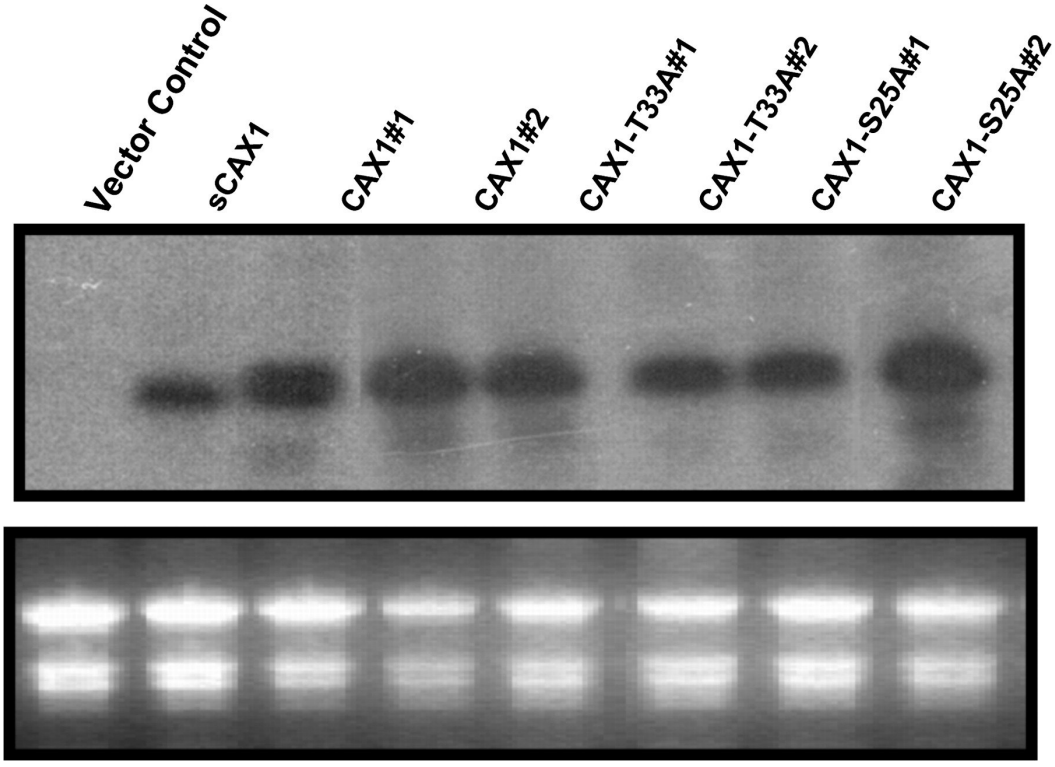


Figure 2.2 Expression of CAX1, CAX1-T33A, and CAX1-S25A in transgenic tobacco plants.

sCAX1, *CAX1*, *CAX1-T33A*, and *CAX1-S25A* transcripts were detected by Northern blot analysis. Ten micrograms of total RNA from tobacco leaves were hybridized with the *CAX1* cDNA probe. Ethidium bromide-stained rRNA (bottom panel) is shown as a loading control.

levels were similar and there was no dramatic copy number difference among the transgenic lines. With these preliminary observations, the CAX1, CAX1-T33A, CAX1-S25A and sCAX1 expressing lines could confidently be compared and contrasted.

Phenotypes of tobacco lines expressing CAX1 variants

Once the appropriate transgenic tobacco lines were identified, phenotypes were compared among CAX1, sCAX1, and the variants previously characterized exclusively in yeast (Hirschi, 1999; Pittman *et al.*, 2002*a*). As reported earlier, transgenic tobacco seedlings that constitutively express the deregulated sCAX1 display hypersensitivity to ion imbalances, such as increased Mg^{2+} and Na^{+} and depleted Ca^{2+} (Hirschi, 1999). As shown in Figure 2.3B, CAX1-T33A-expressing plants were extremely hypersensitive to Ca^{2+}-depleted media in a manner similar to sCAX1-expressing lines. The growth of CAX1- and CAX1-S25A-expressing plants on the Ca^{2+}-depleted media was comparable to vector control plants. In the Na^{+} tolerance growth assays, the same trend was observed. The sCAX1-expressing plants and CAX1-T33A-expressing plants were sensitive to the excess Na^{+} in the media while the CAX1-expressing plants and CAX1-S25A-expressing plants were not (Figure 2.3C). In addition to the ion sensitivity, approximately 16% (6 out of 37) of the CAX1-T33A-expressing plants displayed the tip-burning Ca^{2+}-deficient symptom associated with sCAX1 expression; however, this was not as dramatic as seen in sCAX1-expressing lines which displayed 100% tip-burning symptoms (data not shown). In soil, all CAX1- and CAX1-S25A-expressing plants grew in a manner indistinguishable from vector control lines.

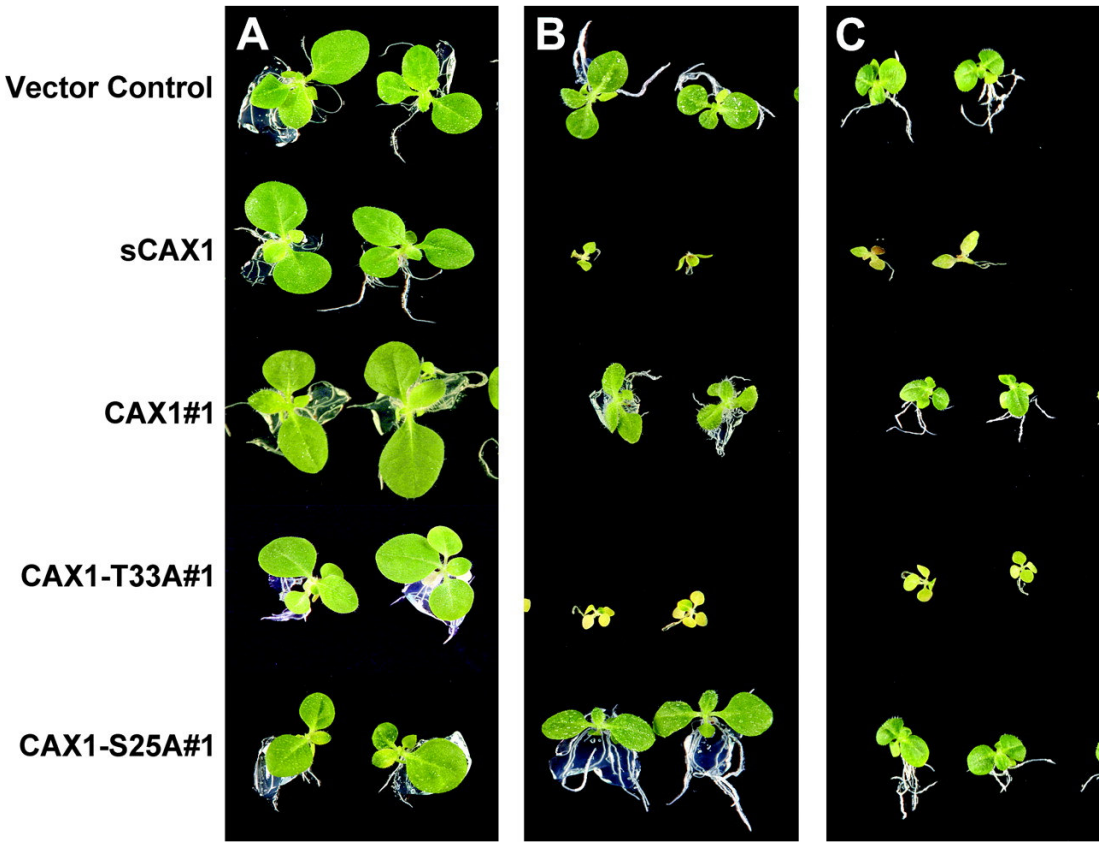


Figure 2.3 Ion sensitivity phenotypes of vector-, sCAX1-, CAX1-, CAX1-T33A-, and CAX1-S25A- expressing tobacco plants.

Plants were transferred 14 d after germination to (A) standard media (0.5x MS), (B) standard media (0.5x MS) without Ca^{2+}, and (C) standard media (0.5x MS) supplemented with 150 mM NaCl. All plants were then grown for 10 d.

Tonoplast Ca^{2+}/H^+ antiport activity from tobacco lines expressing CAX1 variants

It has previously been shown that expression of sCAX1 increases tonoplast Ca^{2+}/H^+ activity at least 30% more in transgenic tobacco roots than in controls (Hirschi, 1999). In addition, the increased Ca^{2+} accumulation was observed primarily in tobacco roots rather than leaves (Hirschi, 1999). As with the ion sensitivity phenotypes, tonoplast Ca^{2+}/H^+ transport activity among vector control, sCAX1-, CAX1-, CAX1-T33A-, and CAX1-S25A-expressing lines were compared. Tonoplast-enriched vesicles were prepared from roots of hydroponically grown transgenic tobacco lines. A steady proton gradient (acid inside vesicles) was established by activation of the Mg^{2+}-ATP-dependent H^+-ATPase. sCAX1-expressing vesicles showed significant Ca^{2+}/H^+ antiport activity. Vector-expressing vesicles showed very low Ca^{2+}/H^+ antiport activity. The CAX1-T33A-expressing line had moderate Ca^{2+} uptake activity compared to that expressing sCAX1, and was approximately 64% of the sCAX1-expressing line (Figure 2.4). However, in the tonoplast vesicles prepared from CAX1-expressing and CAX1-S25A-expressing lines, this uptake assay could not measure any higher Ca^{2+}/H^+ antiport activity than vector control (data not shown). The increase in Ca^{2+}/H^+ antiport activity was consistently measured only in the sCAX1- and CAX1-T33A-expressing lines.

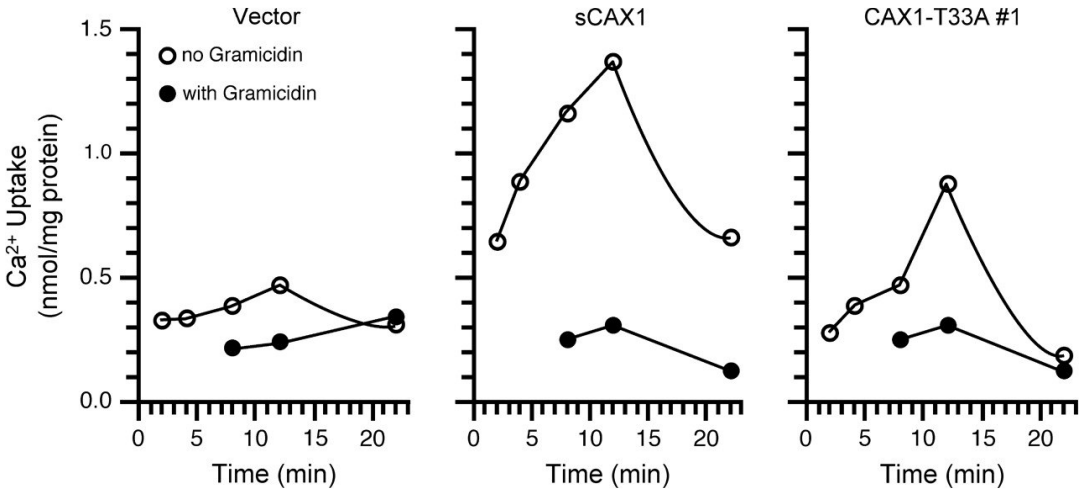


Figure 2.4 Ca^{2+}/H^{+} antiport activity in tonoplast vesicles from vector-, sCAX1- and CAX1-T33A-expressing tobacco root tissue.

Time-courses of ΔpH-dependent 10 µM $^{45}Ca^{2+}$ transport into vacuolar membrane-enriched vesicles prepared from tobacco roots expressing vector alone, sCAX1, or CAX1-T33A as indicated. Ca^{2+} transport was measured in the absence or presence of 5 µM protonophore gramicidin. All time-course experiments were performed in the presence of 1 mM Mg^{2+}ATP. The Ca^{2+} ionophore A23187 (5 µM) was added after 12 min point and significantly dissipated Ca^{2+} accumulation mediated by Ca^{2+}/H^{+} antiport when measured at the 22 min time point. One representative experiment of four experiments is shown.

Metal accumulation in tobacco lines expressing-CAX1 variants

Calcium levels were measured in the transgenic tobacco roots to examine whether altered Ca^{2+}/H^{+} antiport activity impacted the accumulation of Ca^{2+} in tobacco roots. As demonstrated previously (Hirschi, 1999), sCAX1-expressing plants accumulated approximately three times more calcium in root tissue than the vector control lines (Figure 2.5A). A slightly lower amount of calcium was accumulated in the CAX1-T33A-expressing plants as they contained approximately twice as much total calcium in roots as did the vector controls (Figure 2.5A). Meanwhile, the vector controls,

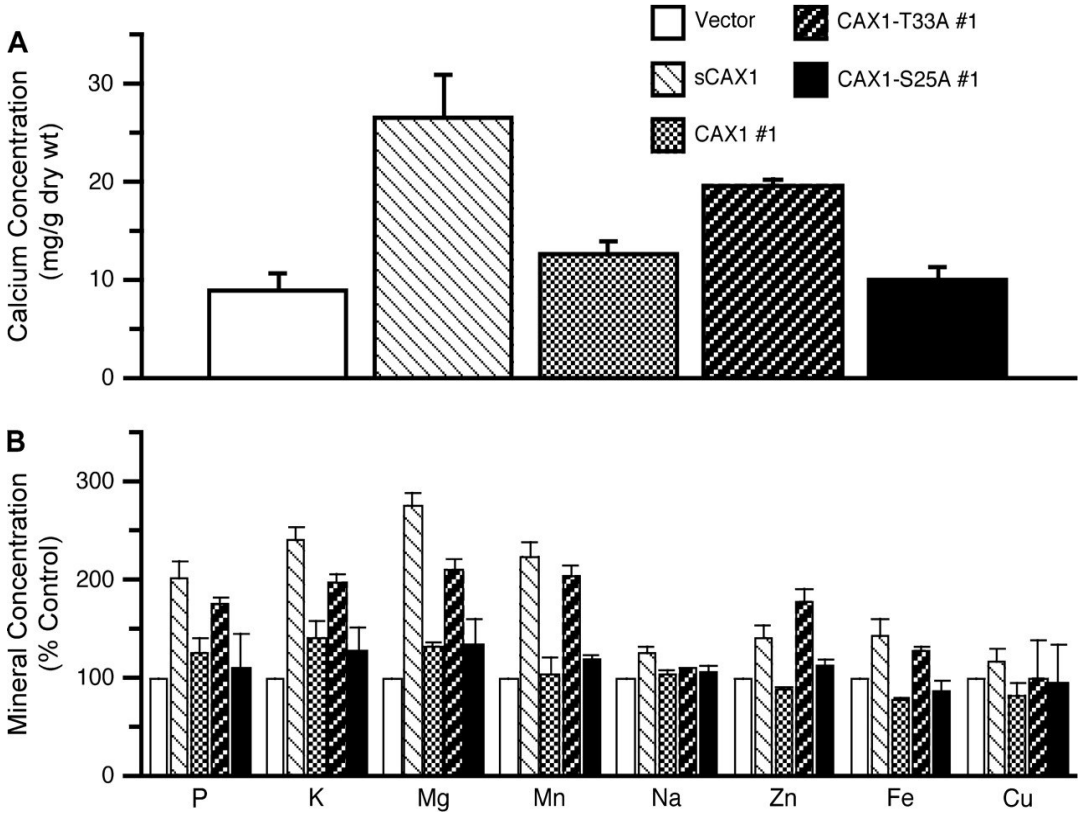


Figure 2.5 Plant ionome.

A) Concentration of calcium in vector-, sCAX1-, CAX1-, CAX1-T33A-, or CAX1-S25A-expressing tobacco root tissue. B) Concentrations of other minerals in vector-, sCAX1-, CAX1-, CAX1-T33A-, or CAX1-S25A-expressing tobacco root tissue. Root tissue of hydroponically grown transgenic tobacco plants were harvested and dried. In (B), values were normalized to concentrations of vector-control tobacco plants. Total mineral concentrations were determined by inductively coupled plasma atomic emission spectrophotometry. Data represents

CAX1- and CAX1-S25A-expressing plants contained similar calcium levels (Figure 2.5A). These results are in general agreement with yeast ionome results presented earlier (Figure 2.1A). Comparison of other mineral profiles revealed that potassium, magnesium, and manganese in sCAX1- and CAX1-T33A- expressing tobacco line were

increased more than 2-fold, while zinc and/or phosphorus content also increased substantially in the sCAX1- and CAX1-T33A-expressing lines (Figure 2.5B). Although concentrations of other minerals were slightly increased in the CAX1- and CAX1-S25A-expressing plants, they were more similar to control plants (Figure 2.5B).

Discussion

Previously, yeast assays have been used to suggest that CAX1 is regulated by an N-terminal autoinhibitory domain. Here, it has been established that these yeast assays provide a robust platform for predicting activity of CAX1 variants in transgenic plants. As far as is known, this type of cross-platform evaluation of N-terminal autoinhibition of plant transporters has not been previously reported. Furthermore, for the first time, it has been confirmed that N-terminal-mediated regulation of a Ca^{2+} transporter occurs *in planta*.

The yeast ionome: a new phenotype to characterize plant transporter function

The ionome provides a rigorous tool to examine the elemental profiles of various samples, be it a plant tissue or a yeast colony (Lahner *et al.*, 2003; Hirschi, 2003; Eide *et al.*, 2005). Given that the expression of many plant transporters in yeast does not generate a visual phenotype, the reproducibility and cost of these yeast ionome assays affords a new phenotype to infer transport function. It has been demonstrated here that measuring the yeast ionome from cells expressing variants of the *Arabidopsis* CAX1 transporter can be a rapid and reproducible inference of transporter function (Figure 2.1). For example, sCAX1 and CAX1-T33A expressing yeast cells both demonstrated high tonoplast Ca^{2+}/H^{+} activity and accumulated significant higher cellular calcium, whereas

yeast cells expressing vector, CAX1 or the CAX1-S25A variant displayed negligible tonoplast Ca^{2+}/H^{+} transport and lower total cellular calcium levels (Pittman *et al.*, 2002*a*; Figure 2.1A). As the mutant yeast strain used is lacking the endogenous tonoplast Ca^{2+}/H^{+} antiporter VCX1, this lack of detectable activity in the vector control and CAX1-/CAX1-S25A-expressing strains was expected. Previously, ion competition and metal-dependent proton transport studies were used to suggest that sCAX1 can transport Ni^{2+}, and possibly Zn^{2+} but not Mn^{2+} (Shigaki *et al.*, 2003, 2005). In these ionome studies sCAX1- and CAX1-T33A- expressing yeast cells also contained significantly increased levels of nickel, and zinc (Figure 2.1B). The increase of nickel and zinc in sCAX1- and CAX1-T33A-expressing lines is in an agreement with our published transport data (Shigaki *et al.*, 2003, 2005; Figure 2.1B). In addition to nickel and zinc, magnesium and iron accumulated to higher levels than control in sCAX1-expressing and T33A-expressing yeast cells (Figure 2.1B). The increase of iron and magnesium could be an indirect result of increased Ca^{2+} transport. However, a previous yeast ionome study suggests that the homeostatic mechanisms that control the levels of different elements are interconnected. For example, changes in nickel levels correlate with changes of magnesium in the yeast ionome (Eide *et al.*, 2005). Indeed, this could explain our observation that the increased magnesium in cells was correlated with increased nickel. While the increased levels of nickel and zinc in the sCAX1 and CAX1-T33A-expressing yeast cells were detectable, the much higher 4-fold increase in calcium in these cells confirms that sCAX1 is predominantly a Ca^{2+} transporter.

Phosphorus, potassium, and manganese levels did not significantly alter in the sCAX1- and CAX1-T33A-expressing yeast cells, yet following expression of these CAX1 variants in tobacco, these three metals were significantly increased. In addition, zinc and iron levels were slightly increased in these lines. Previous analysis has indicated that K^+ is very poorly transported by sCAX1 (Park *et al.*, 2005*b*) while Mn^{2+} and Fe^{2+} are not transported (Shigaki *et al.*, 2003; J Pittman and K Hirschi, unpublished results), although sCAX1 does have some Zn^{2+} transport activity (Shigaki *et al.*, 2005). Previously, increased levels of magnesium, zinc, iron, and manganese were observed in sCAX1-expressing tomato fruits, while phosphorus and potassium were not tested (Park *et al.*, 2005*b*). This is generally consistent with the observations in sCAX1- and CAX1-T33A-expressing tobacco, although the iron and zinc increases are more subtle in tobacco. This may be due to tissue–type differences. The increased accumulation of these metals in the transgenic tobacco plants could be an indirect effect of increased vacuolar Ca^{2+}/H^+ transport activity that may lead to perturbed cytosolic Ca^{2+} levels which, in turn, may regulate other metal transporters (Cheng *et al.*, 2005). This result also highlights the differences that may exist between unicellular yeast and plants with regard to homeostatic regulation for certain metals. However, the trend in calcium content following expression of the various CAX1 constructs was identical between yeast and plant. Thus our experimental approach and results suggest that in heterologous expression studies, the yeast ionome profiles can be used as an initial indicator of *Arabidopsis* transporter function.

CAX1 N-terminal autoinhibition in planta

It has been demonstrated here that transport activity of the full-length CAX1 protein is regulated in transgenic plants compared with significant deregulated transport activity of the N-terminal truncated sCAX1 or the N-terminal point mutant CAX1-T33A. It is suggested that, as in yeast, this down-regulation of *Arabidopsis* CAX1 is due to an autoinhibitory mechanism caused by protein conformation modification at the N-terminus (Pittman *et al.*, 2002*a*). As observed previously, high level expression of sCAX1 in tobacco causes Ca^{2+} deficiency-like phenotypes such as apical burning and hypersensitivity to ion imbalances that are due to excessive and deregulated Ca^{2+} transport into the vacuole by sCAX1 (Hirschi, 1999). When the sCAX1 plants are grown on Ca^{2+}-depleted conditions, the enhanced vacuolar accumulation of trace concentrations of Ca^{2+} acquired by the plant prevent any Ca^{2+} being acquired to other cellular locations where it is essential. The sensitivity to Na^{+} stress is also due to cytosolic Ca^{2+} deficiency caused by excessive vacuolar Ca^{2+} accumulation (Hirschi, 1999; Cheng *et al.*, 2004*b*). By comparison, CAX1 was relatively inactive when expressed in transgenic tobacco plants. For example, none of the Ca^{2+}-deficient symptoms associated with sCAX1 expression were seen in the CAX1-expressing plants (Figure 2.3). Furthermore, increased Ca^{2+}/H^{+} activity was not detected from the vesicles isolated from the CAX1 expressing transgenic roots (Figure 2.4). Thus, the presence of the N-terminal domain of CAX1 appeared to autoinhibit the protein and prevent Ca^{2+} transport activity in tobacco. This also suggests that, unlike when expressed in *Arabidopsis*, mechanisms are not present in tobacco to switch on the autoinhibited *Arabidopsis* CAX1 (see below).

Expression of the N-terminal mutated variants of CAX1 produced phenotypes in tobacco that correlated with their phenotypes in yeast cells. The CAX1-S25A variant, like CAX1, displayed no increased Ca^{2+}/H^{+} activity when expressed in either yeast cells or tobacco plants (Figures 2.1, 2.2, 2.4). Conversely, expressing the CAX1-T33A variant produced phenotypes in both yeast and tobacco (Figure 2.3); however, direct measurement of Ca^{2+} transport activity of CAX1-T33A from the transgenic tobacco plants was lower than sCAX1 (64%) and the percentage of plants displaying apical burning was reduced (16%). Furthermore, the plants expressing-CAX1-T33A variants accumulated less total calcium than sCAX1 lines, but significantly higher levels than CAX1, CAX1-S25A, and vector lines (Figure 2.5A). Previous experiments in yeast found that substitution of Thr-33 with another residue (either Ala, Asp, Ser or Glu) caused a gain of transport activity, leading to the suggestion that this residue plays a role in the proposed autoinhibitory mechanism (Pittman *et al.*, 2002*a*). While substitution of Ser-25 with Ala did not lead to a gain of transport activity, it has been shown previously that a Ser-25 to Asp substitution caused a gain of activity; hence it is proposed that this residue could be a candidate for phosphorylation as a mechanism to activate full-length CAX1 Ca^{2+}/H^{+} antiport activity (Pittman *et al.*, 2002*a*). Further analysis will be required to ascertain the exact functions of these CAX1 N-terminal domain residues.

N-terminal autoinhibition is not unique to CAX transporters. For example, the plant autoinhibited Ca^{2+}-ATPases (ACAs) also have an N-terminal autoinhibitory domain which is equivalent in structure and function to the C-terminal regulatory domain of animal calmodulin-stimulated Ca^{2+}-ATPases (Hwang *et al.*, 2000; Bonza *et*

al., 2004). Studies using a Ca^{2+}-ATPase deficient yeast mutant have demonstrated that plant ACA pumps can functionally complement the mutant only if the N-terminal calmodulin binding/autoinhibitory domain is deleted or if single residues required for autoinhibition have been mutated (Baekgaard *et al.*, 2005). It is clear that calmodulin can stimulate Ca^{2+}-ATPase activity in membrane fractions isolated from plants, and it has also been demonstrated that a peptide corresponding to the autoinhibitory domain of ACA8 can inhibit Ca^{2+}-ATPase activity of a truncated form of the pump in membrane fractions from *Arabidopsis* suspension cells (Luoni *et al.*, 2004). However, the ACA studies have not been extended by functionally expressing the deregulated ACA clones in a plant system to examine this autoinhibition *in planta*, while our work suggests that removal, or modification, of the CAX1 N-terminal domains will activate these transporters when expressed in the plant.

Several signalling molecules have been implicated in regulating plant transporters (Hwang *et al.*, 2000; Cheng *et al.*, 2004*a*). Calmodulin binding to the N-terminus of ACA2 causes activation, whereas the pump can be inhibited by a Ca^{2+}-dependent protein kinase (Hwang *et al.*, 2000). With the CAX transporters similar models have been posited where various regulators, such as the protein kinase SOS2, may either activate or repress transporter function (Cheng *et al.*, 2004*a*, *b*). Our work here suggests the presence of functional N-terminal autoinhibitory domains *in planta* and by extension the cognate signalling molecules. These results may also suggest that the regulatory CAX1 activating proteins, which do not appear to be conserved in yeast, may likewise not be conserved amongst plant species, otherwise it might have been expected

that expression of full-length CAX1 in tobacco would provide transport activity. It will be interesting to test in the future whether co-expression of CAX1 and an activator protein in tobacco causes an increase in Ca^{2+} transport activity. Our working hypothesis therefore is that high level expression of these regulatory modules is an additional means of altering transport function.

Applications for plant improvement

Previous studies have demonstrated that expression of the *Arabidopsis* Ca^{2+}/H^{+} antiporter sCAX1 can be used to increase Ca^{2+} content in several important crops (Park *et al.*, 2004, 2005*a*, *b*). In tomatoes, tempering sCAX1 expression levels by using a weaker promoter resulted in more fruit calcium and a prolonged shelf life, while only modestly compromising plant growth and development (Park *et al.*, 2005*b*). These advantageous alterations in tomato plant growth suggest that further modulation of the expression and activity of a Ca^{2+}/H^{+} transporter can be used to enhance crop growth. For example, expression in tomatoes of CAX4, which appears to have weaker Ca^{2+}/H^{+} antiport activity than sCAX1, provided a modest increase in the calcium content without deleterious phenotypes (Park *et al.*, 2005*b*). The data presented here suggest that, rather than altering expression levels, the N-terminal autoinhibitory domain can be modified to modulate activity. Modifications like the CAX1-T33A mutation in CAX1 could provide important tools for calcium fortification. These variants remain active and thus have higher levels of calcium accumulation (Figure 2.5), but less deleterious effects such as the apical burning associated with sCAX1 expression. Our long-range goal is to engineer CAX variants which are active but produce no deleterious phenotypes.

Conclusion

The N-terminal autoinhibition in the Ca^{2+}/H^{+} transporter CAX1 has been intensively documented using yeast assays. In this study, these studies have been extended to show that N-terminal autoinhibition does indeed occur *in planta*. These findings provide a general platform to modify plant transporter activity.

CHAPTER III

ROOT DEVELOPMENT UNDER METAL STRESS IN *ARABIDOPSIS THALIANA* REQUIRES THE H^+/CATION ANTIPORTER CAX4*

Introduction

Inorganic cations play crucial roles in many cellular and physiological processes in plants and are essential components of plant nutrition (Taiz and Zeiger, 2006). Therefore, the uptake of cations and their redistribution must be precisely controlled to maintain normal physiology and to respond to endogenous and exogenous stimuli in a timely manner. Vacuolar antiporters are important elements in mediating the intracellular sequestration of cations (Busch and Saier, 2002). These antiporters are energized by the proton gradient across the vacuolar membrane and allow the rapid transport of cations into the vacuole. Among such vacuolar antiporters, CAXs (for CAtion eXchanger) have been shown to be involved in a multitude of cellular responses (Shigaki and Hirschi, 2006). However, the role of individual transporters in specific cellular processes has not been completely defined.

The primary cation substrate of CAXs is thought to be Ca^{2+}. Ca^{2+} acts as a secondary messenger in many cellular processes, including hormone-mediated signaling (Cunningham and Fink, 1994; White and Broadley, 2003). CAX-mediated calcium and

---

*This chapter is reprinted with permission from "Root development under metal stress in *Arabidopsis thaliana* requires the H^+/cation antiporter CAX4" by **Mei H., Cheng N.H., Zhao J., Park S., Escareno R.A., J.,Pittman, J.K., and Hirschi, K.D.** (2009). New Phytol. © 2009 with kind permission from Wiley-Blackwell.

metal transport may impact signaling events (Hirschi, 2004; Shigaki and Hirschi, 2006; McAinsh and Pittman, 2009). For example, with regard to auxin, the modulation of cation levels is required for aspects of auxin metabolism and signaling (Felle, 1994; Gehring et al., 1990; Magidin et al., 2003; Rampey et al., 2006) that controls normal development of roots (Muday and Haworth, 1994; Woodward and Bartel, 2005; Wu et al., 2007). In addition, there is evidence that Ca^{2+} plays a role in PIN-FORMED (PIN) transporter-mediated auxin efflux activity. The PIN regulator PINOID (PID), a protein kinase, interacts with Ca^{2+}-calmodulin and appears to be negatively regulated by Ca^{2+}-calmodulin (Benjamins et al., 2003). Thus, it appears reasonable to postulate that CAXs, particularly those expressed in roots, are involved in auxin signaling and metabolism.

In *Arabidopsis*, there are six CAXs, *CAX1* through *CAX6* (Shigaki et al., 2006). Members of the *Arabidopsis CAX* gene family, such as *CAX1*, *CAX2*, and *CAX3* have been characterized at both the molecular and whole plant level (Hirschi et al., 1996; Cheng et al., 2003, 2005; Pittman and Hirschi, 2001; Pittman et al., 2002, 2004; Shigaki et al., 2003). CAX1 was identified by its ability to suppress the Ca^{2+} sensitivity of a yeast mutant strain lacking vacuolar Ca^{2+} transport activity (Hirschi et al., 1996). CAX1 is a low affinity and high capacity Ca^{2+}/H^{+} antiporter, but CAXs may have a wide substrate range (Hirschi, 1999; Shigaki et al., 2003; Pittman et al., 2004; Korenkov et al., 2007b; Edmond et al., 2009). *CAX3* is 77% identical at the amino acid level to *CAX1* and along with *CAX4* is the most closely related gene to *CAX1* (Shigaki and Hirschi, 2000; Shigaki et al. 2006). In *Arabidopsis*, *CAX1* is highly expressed in leaf tissue, and modestly expressed in roots, stems, and flowers (Cheng et al., 2005). The *cax1*

knockout lines exhibit a 50% reduction in total vacuolar Ca^{2+}/H^{+} antiport activity, despite up-regulation of *CAX3* and *CAX4*, and alterations in vacuolar Ca^{2+}-ATPase activity, but the phenotypes of *CAX1* deletion on plant growth are subtle (Cheng et al., 2003). However, when deregulated N-terminal truncated CAX1 (sCAX1) was ectopically expressed in tobacco, the plants displayed dramatic Ca^{2+} deficiency phenotypes, such as tip burning and increased sensitivity to cation imbalances (for review, see Shigaki and Hirschi, 2006). Meanwhile, *CAX3* is expressed mainly in *Arabidopsis* roots and flowers. The *cax3* knockout lines display altered Na^{+}, Li^{+}, and low pH sensitivity (Zhao et al., 2008). However, when CAX3 was expressed in tobacco, no visible phenotypes were observed (Shigaki et al. 2002). *CAX4* has been partially biochemically characterized by heterologous expression in yeast and tobacco to determine cation transport characteristics (Cheng et al., 2002; Korenkov et al., 2007a, 2007b). Expression of full-length CAX4 in tobacco demonstrated that it can mediate proton-coupled Cd^{2+}, Ca^{2+}, Zn^{2+} and Mn^{2+} transport, with highest transport activity for Cd^{2+} (Korenkov et al., 2007b). A yeast-based assay has been informative to elucidate the function of CAX4. For example, when expressed in yeast, mutated variants of CAX4 can transport Ca^{2+} and Cd^{2+}, while addition of the hemagglutinin (HA) epitope tag fused at the N-terminus of CAX4 activates this Ca^{2+}/ H^{+} transporter (Cheng et al., 2002; Park et al., 2005). HA-CAX4 is localized on the vacuolar membrane in both yeast and plant cells (Cheng et al., 2002). Thus we hypothesize that CAX4 is predominantly a vacuolar Ca^{2+} transporter under physiological conditions but can efficiently transport Cd^{2+} when exposed to this metal.

Unlike *CAX1* and *CAX3*, *CAX4* is expressed at very low levels in most tissues (Cheng et al., 2002). Given this weak *CAX4* expression, we initially postulated that CAX4 phenotypes would be difficult to ascertain. However, *CAX4* is moderately expressed in roots (Cheng et al., 2002). With this relatively root-specific expression of *CAX4* in mind, we were interested to investigate the role of this gene in *Arabidopsis* root development to reveal specific functions of vacuolar Ca^{2+}/H^{+} transporters in plant growth and development.

In this study we dissect the function of CAX4 *in planta*. We show the expression pattern of *CAX4* in *Arabidopsis* roots and identify stimuli which modulate gene expression. We report the isolation of plants perturbed in *CAX4* expression and some of the phenotypes associated with altered expression. Collectively, these findings offer insights into the physiological function of CAX4 in root development and stress responses.

Materials and Methods

Plasmid DNA constructs and plant transformation

To make a transcriptional fusion to the GUS reporter gene, the *CAX4* promoter region 740 bp upstream of the ATG start codon was amplified from *Arabidopsis* genomic DNA by PCR using the following primers: forward primer 5'-GCCAAGCTTGGGAGGCCTAAAGCAAATCAC-3' and reverse primer 5'-GCCGGATCCCTTCTTCGTTGACTCTCTTTG-3' and cloned into the pBI121 vector to replace the cauliflower mosaic virus 35S promoter.

The *CAX4* and triple HA epitope-tagged *CAX4* (*HA-CAX4*) cDNA constructs have been previously described (Cheng et al., 2002). *CAX4* and *HA-CAX4* were subcloned into the plant expression vector pBI121 by replacing the β-glucuronidase gene, which is driven by a cauliflower mosaic virus 35S promoter.

For modification of *CAX4* expression by RNAi, a 1.2 kb- cDNA from *CAX4* was used. This cDNA fragment was amplified by PCR using the following primer paris: forward primer 5'-GCGTTAATTAAGGCGCGCCGGAAGAGATCGGATCTGAAA-3' and reverse primer 5'GCGGGATCCATTTAAATGCAATGAGCAGCATCACGAAGCTC-3'. The forward primer contained PacI and AscI restriction site and the reverse primer contained BamHI and SwaI site. This *CAX4* fragment was cloned into the pFGC5941 binary vector (Kerschen et al., 2004) in both sense and antisense orientations and used to transform wild type (Columbia ecotype) plants.

The recombinant plasmids, the CAX4 RNAi construct, and empty vector controls were transformed into *Agrobacterium tumefaciens* GV3101 (Sambrook et al., 1989) or LBA4404 (Invitrogen, CA, USA). These strains were used to transform *Arabidopsis* Col-0 using the floral dip method (Clough and Bent, 1998). Transgenic tobacco (*Nicotiana tabacum* cv KY14) overexpressing CAX4 were previously described (Korenkov et al., 2007a, 2007b).

Histochemical assay of GUS gene expression

Histochemical assays for β-glucuronidase (GUS) activity in the T3 generation of *Arabidopsis* transgenic plants harboring *CAX4::GUS* were performed according to the protocol described previously (Cheng et al., 2003).

Isolation of cax4 and creation of CAX4 RNAi lines

To isolate a *cax4* null allele, the line SM_3.16922, carrying a d*Spm* transposon insertion in *CAX4* (ecotype Col-0) was obtained from the SLAT collection (Sainsbury Laboratory *Arabidopsis thaliana* Transposants) (Tissier et al., 1999). Homozygous plants from T3 generation were obtained by PCR screening using *CAX4*-specific (P1 and P2) and d*Spm*-sepcific (P3) primers, the d*Spm*-sepcific primer, P3, 5'-TACGAATAAGAGCGTCCATTTTAGAGTGA-3', and the *CAX4* reverse primer, P2, 5'-TGATACCCTTAAACATAACTTACCTTTTCGT-3', were used to screen for the *cax4-1* allele. The *CAX4* forward primer, P1, 5'-AATTGTTGGTAATGCAGCTGAGCAT-3', and the *CAX4* reverse primer (as above), were used to amplify the wild-type *CAX4* gene. Two sets of *CAX4* specific primers were used in RT-PCR to confirm lack of transcript in the *cax4-1* allele: the *CAX4* set 1 forward primer, CAX4F1, 5'- ATCGGCGTCGTCGTTGATAAGGAA-3', and the *CAX4* set 1 reverse primer, CAX4R1, 5'- ACAGCGCCAGATACAACAACATGC-3'; the *CAX4* set 2 forward primer, CAX4F2, 5'- GCATGTTGTTGTATCTGGCGCTGT-3', and the *CAX4* set 2 reverse primer, CAX4R2, 5'-CAGCATGCTCAGCTGCATTACCAA-3'.

Northern blot analysis

For Northern blot analysis, total RNA was extracted from transgenic *Arabidopsis* and tobacco flowering plants overexpressing *CAX4*, blotted and hybridized with ^{32}P-labeled *CAX4* gene specific probes as previously described (Cheng et al., 2003).

Western blot analysis

For Western blot analysis, total microsomal protein was extracted from transgenic *Arabidopsis* overexpressing HA-CAX4 and wild type *Arabidopsis* (Randall et al., 1986). Immunoblot analysis was preformed and the HA epitope was detected as described previously (Pittman and Hirschi, 2001).

Plant materials and growth conditions

Arabidopsis Col-0 was used in this study. Wild-type, *cax4-1, CAX4* RNAi and *cax4-1/HA-CAX4* seeds were surface sterilized, germinated, and grown on the one half-strength MS medium (Murashige and Skoog, 1962). All plates were sealed with paper surgical tape and incubated at 22°C under continuous cool-fluorescent illumination. For the ion sensitivity assays, five-day-old *Arabidopsis* wild type, *cax4-1, CAX4* RNAi and *cax4-1/HA-CAX4* seedlings grown under normal conditions were transferred onto the half-strength MS media and the half-strength MS media supplemented with various metal ions. The ion sensitivity assay for the transgenic tobacco plant was preformed as previously described (Hirschi, 1999).

Preparation of membrane vesicles and transport measurements

For the measurement of Ca^{2+} uptake, vacuole-enriched membrane vesicles were prepared from root tissue obtained from two-week-old Col-0, *cax1-1* and *cax1-1*/*CAX4*

plants cultured in Gamborg's B5 medium and pretreated with 100 mM $CaCl_2$ for 18 hours before harvest. Membrane vesicles preparation and Ca^{2+} uptake assay were performed as described previously (Hirschi, 1999; Cheng et al., 2003)

Results

CAX4 expression in Arabidopsis *roots*

RT-PCR analysis indicates that *CAX4* transcripts can be detected in all *Arabidopsis* tissues analyzed, including stems, flowers, leaves, siliques and roots (Cheng et al., 2002). However, the expression of *CAX4* is the highest in root tissue. This root-specific expression for *CAX4* is also observed in publically available microarray data. To further study the expression pattern of *CAX4* in roots, histochemical analysis of transgenic plants harboring the *CAX4::GUS* reporter was performed. A 740 bp fragment containing the *CAX4* promoter was fused to the *GUS* gene. The *CAX4::GUS* construct was introduced into wild-type *Arabidopsis* plants and GUS activities of T3 plants were analyzed at different root growth and developmental stages. At a very early stage (one day after germination), the GUS signal was detected in root tip as well as hypocotyls and the base of cotyledon (data not shown). Three days after germination, the GUS signal was visualized in the primary root apex and lateral root primordium (Figure 3.1). When seedlings were grown on MS media, the *CAX4::GUS* signal was weak (Figure 3.1), which is consistent with previous findings (Cheng et al., 2002). However, when seedlings were exposed to elevated levels of Ni^{2+}, Mn^{2+}, or depleted of Ca^{2+}, the *CAX4::GUS* signal was enhanced in the primary root apex, in the lateral root primordium, and in the primary root elongation zone. These same conditions also

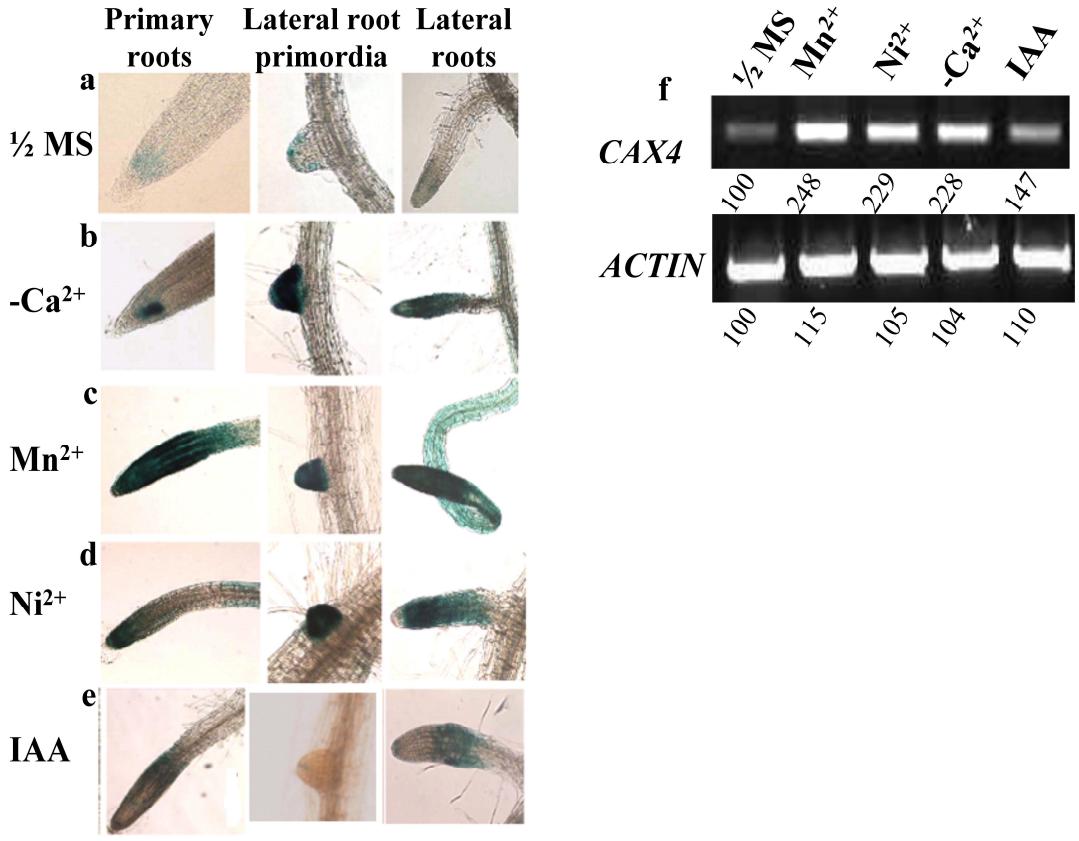


Figure 3.1 *CAX4* expression in *Arabidopsis* roots.

a. *CAX4* promoter::GUS expression in transgenic Arabidopsis roots. a. *CAX4::GUS* expression in 7-d-old seedlings. Note the preferential GUS staining in the root meristem. b. GUS staining in the primary root apex, lateral root primordia, and the lateral root under Ca^{2+} depleted condition. c. GUS staining in the primary root apex, lateral root primordia, and the lateral root elongation zone after treatment with 2 mM Mn^{2+}. d. GUS staining in the primary root apex, lateral root primordia, and the lateral root elongation zone after treatment with 0.1 mM Ni^{2+}. e. GUS staining in the primary root apex, lateral root primordia, and the lateral root elongation zone after treatment with 20 nM IAA. All stress conditions were administered for 2 days. f. *CAX4* transcripts were detected by RT-PCR in total RNA from roots of wild type seedlings treated for 2 days with the following conditions: ½ MS media, ½ MS+2 mM Mn^{2+}, ½ MS+0.1 mM Ni^{2+}, depletion of Ca^{2+} from ½ MS, and ½ MS+25 nM IAA, respectively. Top panel in f, a 414-bp *CAX4*-specific fragment was amplified by RT-PCR. Bottom panel in f, a 457-bp *ACTIN* fragment was amplified by RT-PCR as an internal control. The numbers indicate the relative intensities of PCR bands in percentages as compared with control (grown on ½ MS as a control).

increased *CAX4* expression in the lateral root apex and throughout the lateral roots (Figure 3.1). Cd^{2+} treatment did not enhance *CAX4::GUS* levels. In addition, exogenous IAA induced the *CAX4::GUS* expression in the primary root apex and lateral root elongation zone (Figure 3.1). RT-PCR analysis confirmed the GUS results, these experiments were performed using RNA extracted from roots of wild type plants that were grown on the same metal and hormone treatments for which the *CAX4::GUS* expressing plants were tested (Figure 3.1f).

Identification of a CAX4 mutant allele and CAX4 RNAi lines

To investigate the physiological function of CAX4 *in planta*, we used a transposon insertion line, SM_3.16922. The transposon insertion was confirmed by PCR with a combination of gene-specific (P1 and P2) and transposon-specific primers (P3) as shown in Figure 3.2a. A homozygous line was isolated by PCR analysis and was termed *cax4-1*. The *cax4-1* line contains the transposon insertion in the 8th intron, 2226 nucleotides downstream of the start codon of the *CAX4* gene (Figure 3.2a). RT-PCR was performed to determine the *CAX4* transcript levels in *cax4-1*. As shown in Figure 2b, no detectable *CAX4* expression was observed in the *cax4-1* plants, indicating the expression of *CAX4* was completely disrupted by the transposon insertion. Because only one *cax4* line was obtained, we generated *Arabidopsis* lines harboring RNA interference (RNAi) constructs for *CAX4* to down-regulate its expression. The effect of the RNAi on *CAX4* transcript accumulation was assessed by RT-PCR. Two RNAi lines with clearly decreased expression of *CAX4* were employed for further studies (Figure 3.2d). Lines of *cax4-1* expressing the deregulated version of *CAX4* (*HA-CAX4*) (Cheng et al., 2002)

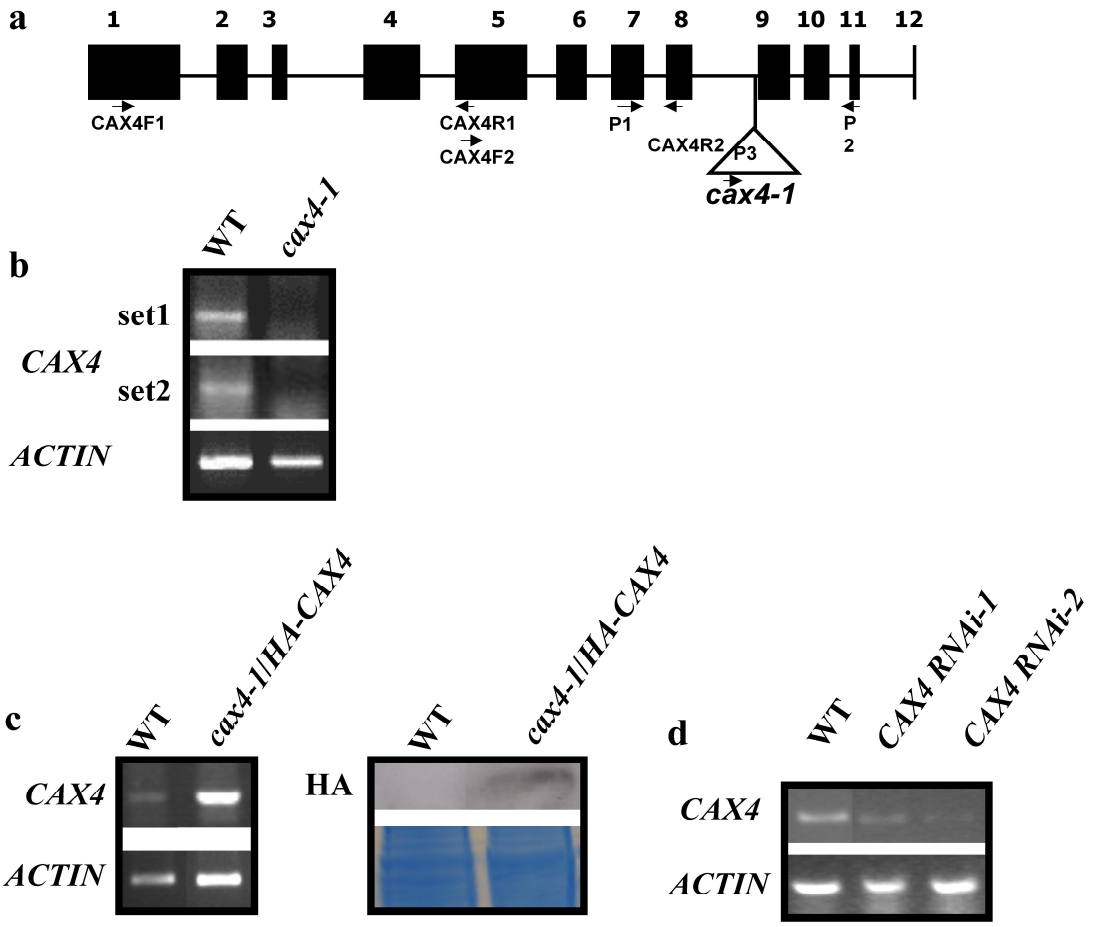


Figure 3.2 Analysis of a *cax4* knockout allele and *CAX4* RNAi lines.

a. Diagram of the *CAX4* gene depicting the site of the transposon insertion. The approximately 3kb of genomic *CAX4* DNA is represented by 11 introns (lines) and 12 exons (boxes). The triangle indicates the sites of the transposon location. b. RT-PCR analysis of *CAX4* expression in wild-type and *cax4-1*. c. RT-PCR and Western analysis of CAX4 expression in wild-type and *cax4-1/HA-CAX4* lines. d. RT-PCR analysis of *CAX4* expression in wild-type, *CAX4 RNAi-1*, and *CAX4 RNAi-2*. Total RNA was extracted from the roots of 3-week-old wild-type, *cax4-1*, *cax4-1/HA-CAX4, CAX4 RNAi-1,* and *CAX4 RNAi-2* seedlings. Top panel in b and d, a 520-bp *CAX4*-specific fragment was amplified by RT-PCR. Middle panel in b, a 430-bp *CAX4*-specific fragment was amplified by RT-PCR. Bottom panel in b, c, and d, a 457-bp *ACTIN* fragment was amplified by RT-PCR as an internal control. Total microsomal proteins were extracted from 3-week-old wild type and *cax4-1/HA-CAX4* seedlings. Top panel in c, a band around 49 kD, which is the expected protein size of CAX4, was detected. Bottom panel in c, the Coomassie Blue G-250 (Invitrogen) stained protein gel was shown as the loading control.

were also generated so that any *CAX4* loss-of-function phenotype could be rescued. Three transgenic lines (*cax4-1*/*HA-CAX4*) harboring *HA-CAX4* under the control of the CaMV 35S promoter were generated. The high level of *HA-CAX4* expression in *cax4-1* was confirmed using both RT-PCR and Western blot analysis using an antibody against the HA epitope (Figure 3.2c).

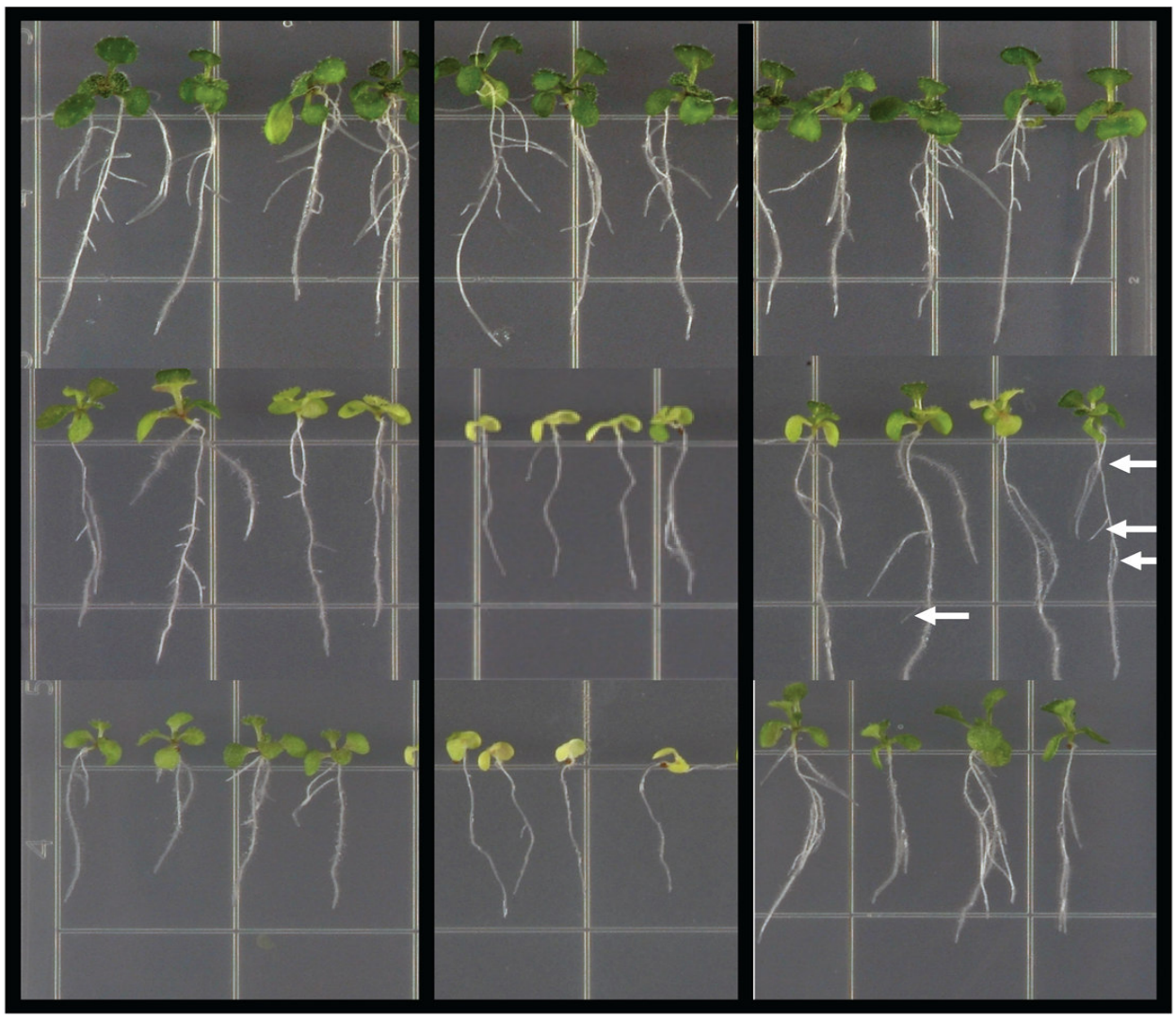
Defects in cax4 root development under stress conditions

We examined *cax4-1* and *CAX4* RNAi transgenic lines under a wide range of conditions comparing their growth and development to controls. Given that cations modified *CAX4* expression, we tested *cax4-1* and *CAX4* RNAi transgenic lines on Ca^{2+} depleted, Ni^{2+}, Cd^{2+}, Ca^{2+} or Mn^{2+} containing medium. When *cax4-1* and *CAX4* RNAi plants were germinated and grown on Cd^{2+} and Mn^{2+} media, altered root growth and development was observed in the *cax4-1* and *CAX4* RNAi lines compared to wild type (Figure 3.3b). Under these conditions, the *cax4-1* and *CAX4* RNAi seedlings were much smaller than control seedlings. Both primary root length and lateral root number were significantly reduced (Figure 3.3a). The most severe phenotype of *cax4-1* was observed on Cd^{2+} medium, where the development of lateral roots was almost completely disrupted (reduced 70% compared to wild type on 2µM Cd^{2+}) (Figure 3.3b). In addition, on the Cd^{2+} containing medium, the cotyledons of the *cax4-1* and *CAX4* RNAi seedlings became yellow and a marked reduction in chlorophyll was observed (Figure 3.3). On Ca^{2+} depleted medium, Ni^{2+}- or Ca^{2+}-containing medium, the growth and development of *cax4-1* and *CAX4* RNAi transgenic lines were similar to control plants. Likewise, when grown on soil, no differences from controls were observed (data not shown). In all

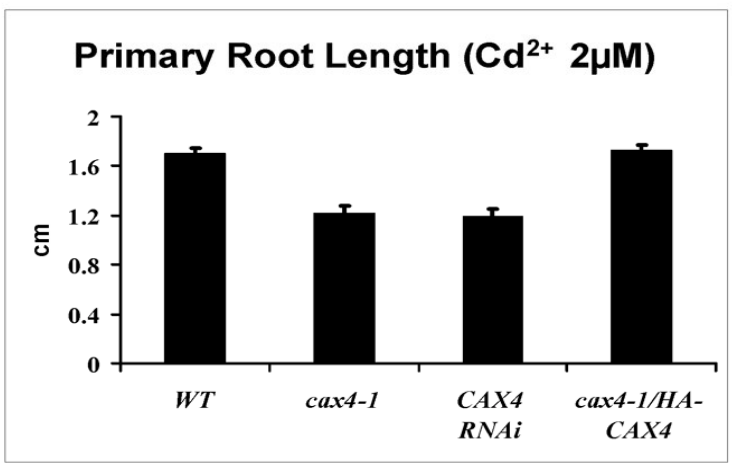
Figure 3.3 Ion sensitivity of *cax4-1* and *cax4-1/HA-CAX4* seedlings.

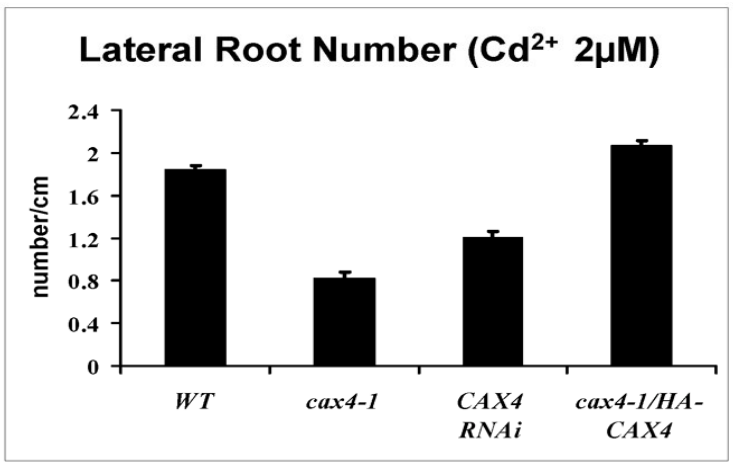
a. In each panel, four wild-type plants are shown on the left, four *cax4-1* plants are shown in the middle, and four *cax4-1/HA-CAX4* plants are shown on the right. All photographs are representative of >100 plants grown in each condition. Top panel, seeds were germinated on ½ MS medium and grown for 10 d. Middle panel, seeds were germinated on ½ MS containing 2 μM Cd^{2+} and grown for 10 d. Bottom panel, seeds were germinated on ½ MS containing 5 μM Cd^{2+} and grown for 10 d. b. Root growth measurements. Primary roots and lateral roots of seedling grown on the ½ MS containing 2 μM Cd^{2+}, 5 μM Cd^{2+} or 0.5 mM Mn^{2+} were measured and counted after 10 d. Error bars represent standard error of the means (n≥12). *cax4-1* had significantly shorter primary roots on 0.5 mM Mn^{2+} (P≤0.0003), 2 μM Cd^{2+} (P≤0.0001) or 5 μM Cd^{2+} (P≤0.003) and fewer lateral roots than wild type on 0.5 mM Mn^{2+} (P≤0.0001), 2 μM Cd^{2+} (P≤0.005) or 5 μM Cd^{2+} (P≤0.0001) in two-tailed t-tests assuming unequal variance. The *CAX4* RNAi line also had significantly shorter primary roots on 0.5 mM Mn^{2+} (P≤0.0001), 2 μM Cd^{2+} (P≤0.0001) or 5 μM Cd^{2+} (P≤0.009) and fewer lateral roots than wild type on 0.5 mM Mn^{2+} (P≤0.0001), or 5 μM Cd^{2+} (P≤0.0006) in two-tailed t-tests assuming unequal variance.

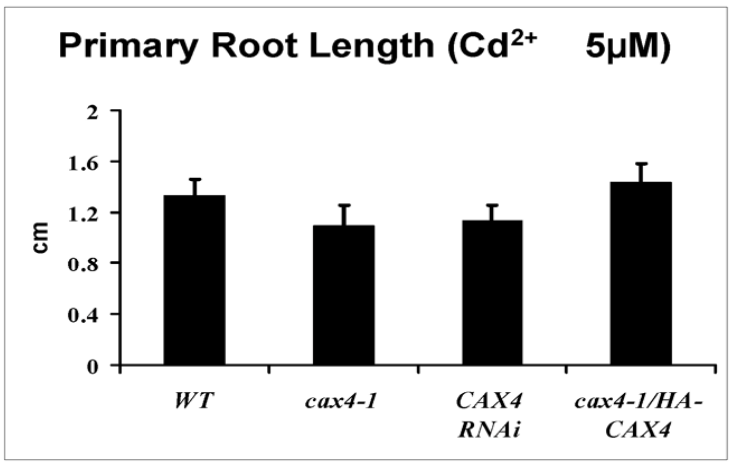
a

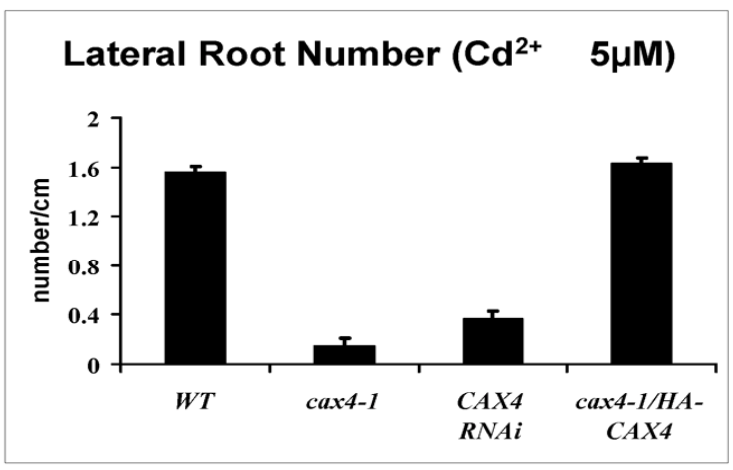

WT
cax4-1
cax4-1/HA-CAX4
½ MS
2μM Cd2+
5μM Cd2+

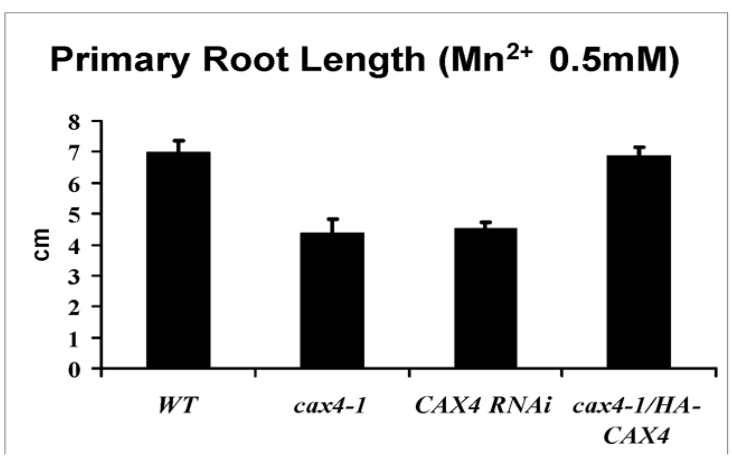
b

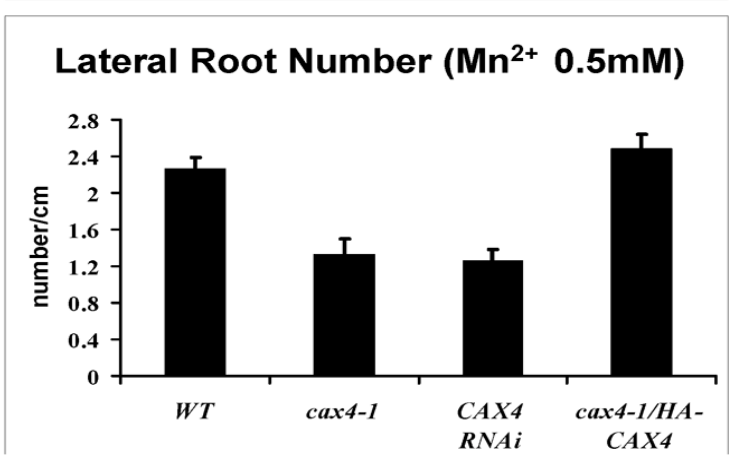

Primary Root Length (Cd2+ 2μM)
cm
2
1.6
1.2
0.8
0.4
0
WT
cax4-1
CAX4 RNAi
cax4-1/HA-CAX4


Lateral Root Number (Cd2+ 2μM)
number/cm
2.4
2
1.6
1.2
0.8
0.4
0
WT
cax4-1
CAX4 RNAi
cax4-1/HA-CAX4


Primary Root Length (Cd2+ 5μM)
cm
2
1.6
1.2
0.8
0.4
0
WT
cax4-1
CAX4 RNAi
cax4-1/HA-CAX4


Lateral Root Number (Cd2+ 5μM)
number/cm
2
1.6
1.2
0.8
0.4
0
WT
cax4-1
CAX4 RNAi
cax4-1/HA-CAX4


Primary Root Length (Mn2+ 0.5mM)
cm
8
7
6
5
4
3
2
1
0
WT
cax4-1
CAX4 RNAi
cax4-1/HA-CAX4


Lateral Root Number (Mn2+ 0.5mM)
number/cm
2.8
2.4
2
1.6
1.2
0.8
0.4
0
WT
cax4-1
CAX4 RNAi
cax4-1/HA-CAX4

cases where *cax4-1* growth was perturbed, expression of *HA-CAX4* was able to restore the growth changes that were attributed to reduced *CAX4* expression (Figure 3.3a).

CAX4 *expression in cax1-1 mutant plants partially restores* Ca^{2+}/H^{+} *antiport activity*

To determine whether CAX4 functions in *Arabidopsis* Ca^{2+} transport, CaMV 35S::*CAX4* was expressed in *cax1-1* mutant plants. This mutant background is ideal for measuring vacuolar Ca^{2+}/H^{+} antiport activity because the mutant lines have 50% less activity than control plants (Cheng et al., 2003). Consistent with our previous findings, the vacuolar Ca^{2+}/H^{+} antiport activity of *cax1* membrane vesicles was decreased by 61% compared to wild type controls (Figure 3.4). In contrast, the *cax1-1* lines expressing CaMV 35S::*CAX4* displayed only a 33% decrease in vacuolar H^{+}/Ca^{2+} antiport activity (Figure 3.4).

Expression of CAX4 in transgenic Arabidopsis *and tobacco induces ion sensitivity*

We postulate that CAX4 has similar biochemical functions to CAX1 in plants. To investigate this possibility, we compared the phenotypes of plants expressing *CAX4* with those expressing *sCAX1* from previous studies (Hirschi, 1999; Cheng et al., 2003). We used independently transformed tobacco and *Arabidopsis* plants, which expressed high levels of *CAX4* under the control of the CaMV *35S* promoter. As shown in Figure 3.5a and 3.5c, expression of *CAX4* in five independent transgenic tobacco and four independent *Arabidopsis* lines was confirmed by Northern blot and the lines with highest expression (tobacco T1-35 and *Arabidopsis* F3-5-8) were used for further studies. As described earlier, constitutive expression of the deregulated *sCAX1* in tobacco plants causes dramatic changes in plant growth and stress responses (Hirschi, 1999). The same

conditions that were previously tested for s*CAX1*-expressing tobacco plants were used in this study to assay *CAX4*-expressing transgenic tobacco lines. The *CAX4*-expressing

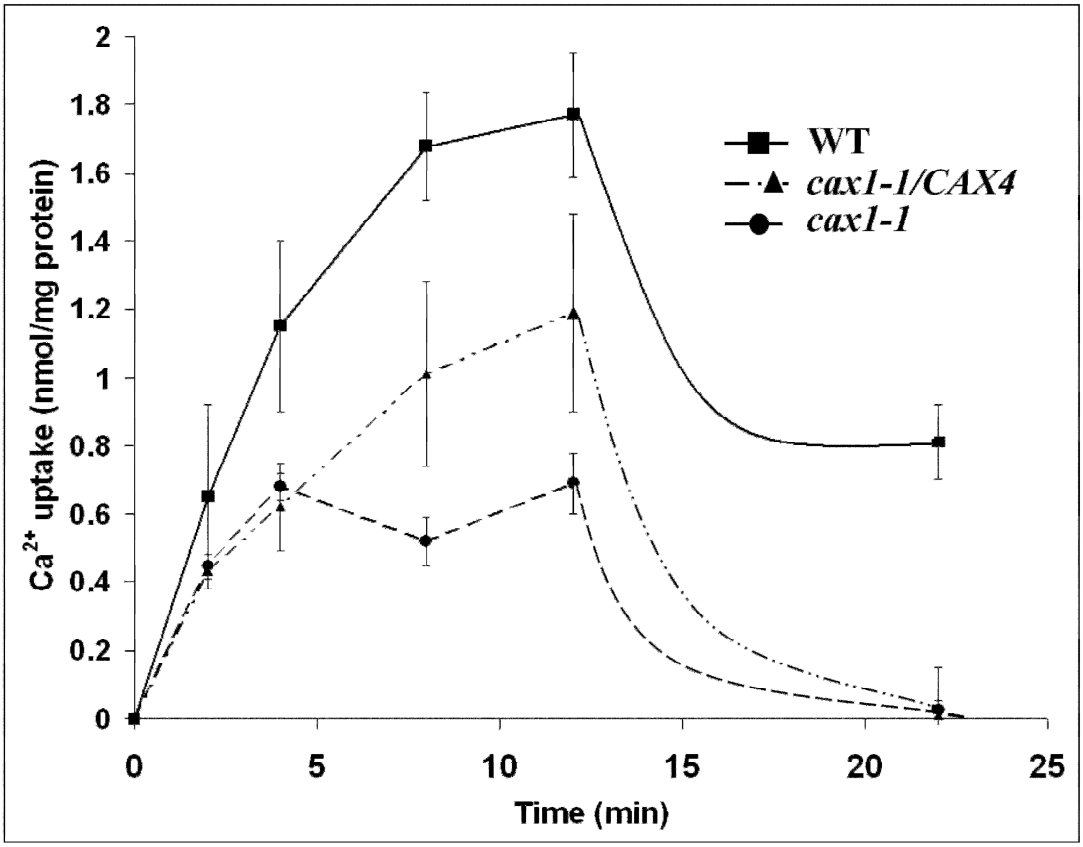


Figure 3.4 Ca^{2+} uptake into vacuole enriched membrane vesicles from Ca^{2+} treated wild-type (squares), *cax1-1*(circles), and *cax1-1/CAX4* (triangles) root tissues.

Time courses of Ca^{2+} uptake by Mg^{2+}-ATP–energized vacuole-enriched membrane were determined in the presence of 0.1 mM NaN_3, 10 mM KCl, 1 mM ATP, and 10 µM $^{45}Ca^{2+}$. ΔpH-dependent Ca^{2+}/H^+ antiport activity was confirmed by adding 5 µM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) in uptake reaction as a negative control and net H^+/Ca^{2+} antiport activity was shown after the subtraction of the FCCP background values. The Ca^{2+} ionophore A23187 (5 µM) was added after 12 min point and dissipated Ca^{2+} accumulation mediated by Ca^{2+}/H^+ antiport when measured at the 22 min time point. All results are means ± SE of experiments from three independent membrane preparations.

tobacco lines, like *sCAX1*-expressing lines, showed apical burning and necrotic lesions on their leaves in media containing Mg^{2+}, Mn^{2+} or depleted of Ca^{2+} (Figure 3.5b). These growth conditions did not affect the controls. *Arabidopsis* lines expressing CaMV *35S*::*CAX4* also showed severe necrotic lesions in leaves when grown in the presence of increased levels of Mg^{2+} (Figure 3.5d). Previous studies have demonstrated that adding Ca^{2+} ameliorates the toxicity of Mg^{2+} in the medium in *sCAX1*-expressing tobacco lines (Hirschi, 1999). In the similar manner, the increased sensitivity of *CAX4*-expressing tobacco or *Arabidopsis* plants was alleviated with the addition of Ca^{2+} (Figure 3.5b and 3.5d).

cax4 is more sensitive to exogenous auxin

CAX4 is highly expressed in areas of the root where auxin may influence growth and development. Furthermore, plants altered in *CAX1* expression have altered auxin perception phenotypes (Cheng et al 2003). We thus sought to address the role of CAX4 in auxin mediated root growth. Figure 3.6 shows that the root elongation of *cax4-1* plants was inhibited by indole-3-acetic acid (IAA) and by synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) in plants (data not shown). The inhibition of root elongation was more pronounced in *cax4-1* lines than *CAX4* RNAi lines. In all cases, expression of *HA-CAX4* caused *cax4-1* line to grow in a manner indistinguishable from controls (Figure 3.6). These *cax4* phenotypes appeared to be auxin specific. We tested the *cax4-1* line and *CAX4* RNAi lines on media containing cytokinin, gibberellins, abscisic acid or brassinolides, and no visible growth differences were observed between *cax4-1* and control plants (data not shown).

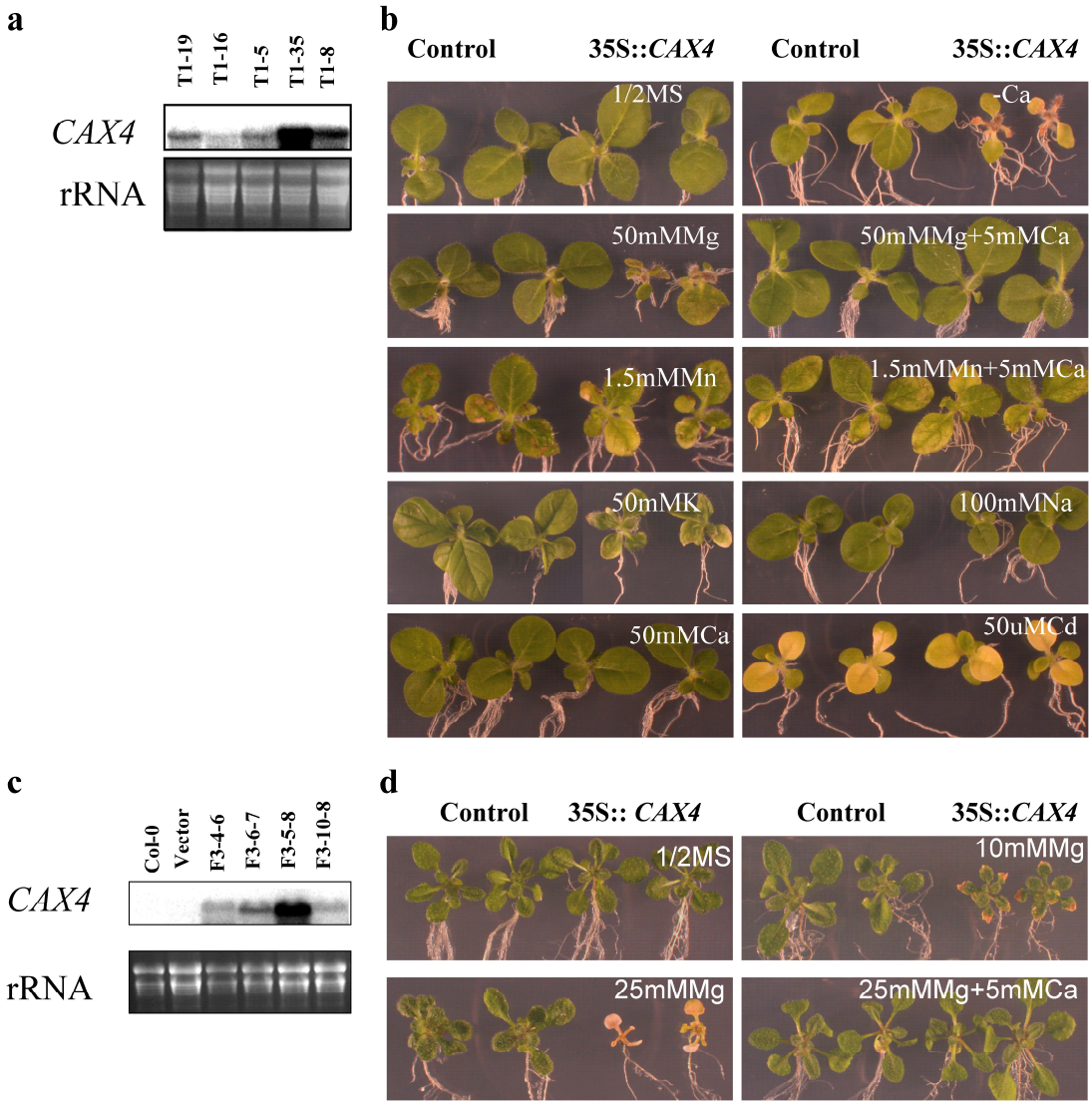


Figure 3.5 Expression of *CAX4* in transgenic plants and ion sensitivity of *CAX4*-expressing plants.

a and c. Ten micrograms of total RNA extracted from fully expanded leaves of 6-week-old T2 tobacco plants or 2-week-old Arabidopsis plants was analyzed by RNA gel blotting. The blot was hybridized with the *CAX4* cDNA probe. Ethidium bromide–stained rRNA before transfer is shown at bottom. b. Two vector plants are shown at left and two *CAX4*-expressing tobacco plants (*35S::CAX4*) at right. 10-day-old tobacco seedlings grown in ½ MS media and transferred to ½ MS or ½ MS containing the following metals: Mg^{2+}, Mn^{2+}, K^{+}, Na^{+}, Ca^{2+}, Cd^{2+} or Ca^{2+} depletion (concentrations of metals are indicated in figure) and grown for 10 days. d. Two vector control plants are shown at left and *CAX4*-overexpressing Arabidopsis plants (*35S::CAX4*) plants at right. 5-day-old Arabidopsis plants transferred to ½ MS media or ½ MS containing different metals (concentrations of metal are indicated in figure) and grown for 5 days.

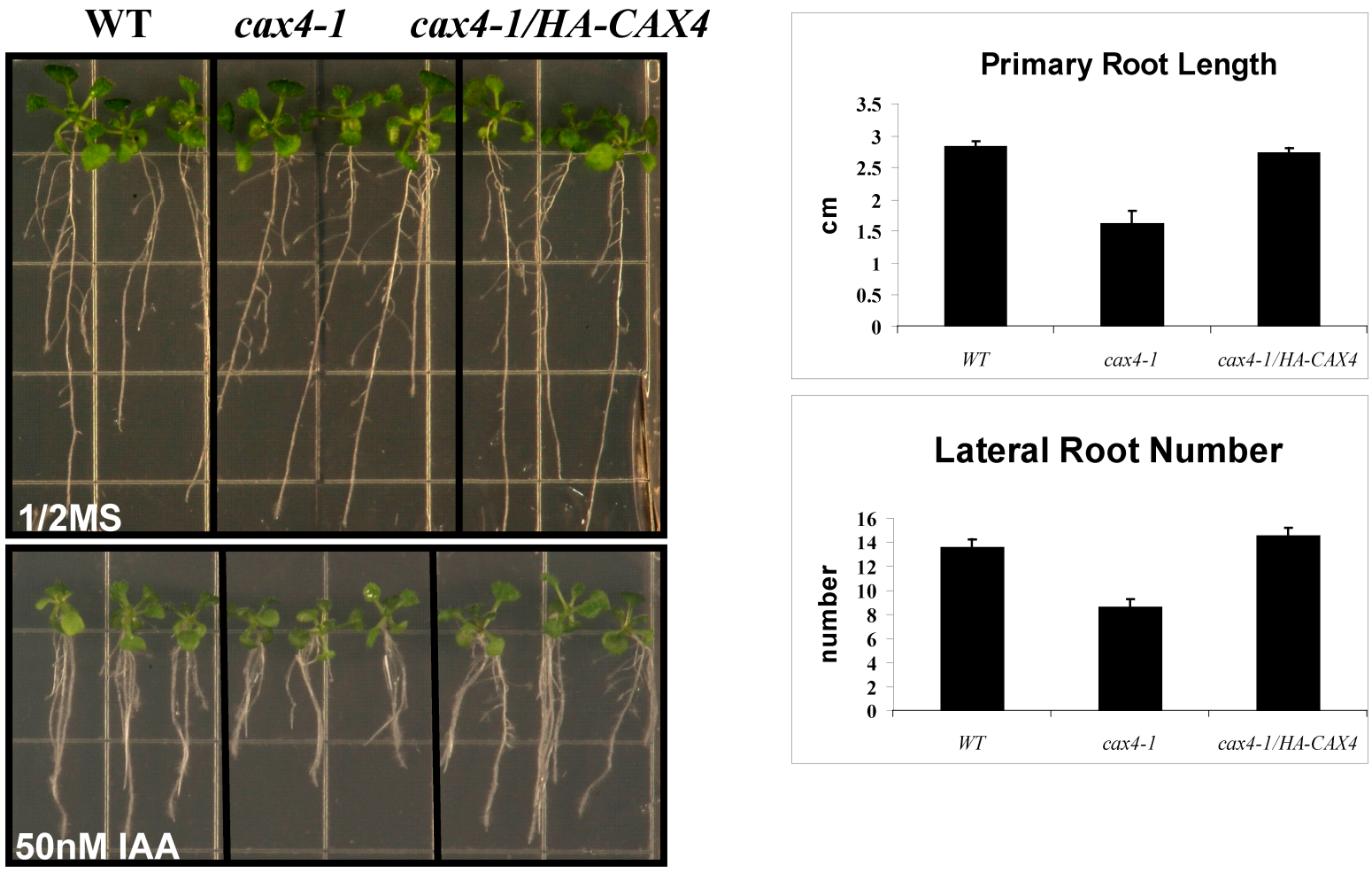


Figure 3.6 Root elongation inhibition by exogenous auxin.

a. In each panel, three wild-type plants are shown on the left, three *cax4-1* plants are shown in the middle, and three *cax4-1/HA-CAX4* plants are shown on the right. All photographs are representative of >100 plants grown in each condition. a. Top panel, seeds were germinated on ½ MS medium and grown for 10d. Middle panel, seeds were germinated on ½ MS and grown for 6 days, then transferred to ½ MS containing 50 nM IAA and grown for 4 d. b. Root growth measurements. Primary roots and lateral roots of seedling grown on the ½ MS containing 50 nM IAA were measured and counted after 4 days. Error bars represent standard error of the means (n≥14). *cax4-1* had significantly shorter primary roots ($P \leq 0.001$) and less lateral roots ($P \leq 0.001$) than wild type in two-tailed t-tests (assuming unequal variance).

To determine if the growth inhibition in *cax4* is due to the altered auxin responses, histochemical analysis of the *DR5::GUS* auxin reporter in wild type and *cax4* roots was performed. *DR5* is a synthetic auxin-responsive promoter that consists of a 7X repeat of the ARF site (AuxRE) from the soybean *GH3* promoter and a CaMV *35S* minimal promoter fused to the GUS-encoding reporter gene (Ulmasov et al., 1997). In *Arabidopsis*, *DR5::GUS* is sensitive to auxin in a dosage dependent manner, and its *DR5::GUS* signal was restricted to the root apical meristem, quiescent center and the provascular tissue in both wild type and *cax4-1* plants (Figure 3.7). When exposed to exogenous IAA or 2,4-D for 24 hours or 48 hours, *DR5::GUS* expression in wild type was increased (Ulmasov et al., 1997; Sabatini et al., 1999, Fig. 7a), while, in the *cax4-1* line, *DR5::GUS* expression was unchanged (Figure 3.7a). This result suggests that CAX4 may be required for auxin responses of *DR5::GUS* expression. However, there is possibility that effects of altered *CAX4* levels on *DR5::GUS* expression are indirect through *CAX4*-mediating metal ion sequestration. To test whether metals are involved in these auxin responses, the *DR5::GUS* expressions were examined under different metal treatments. Interestingly, when grown on the media containing Cd^{2+}, *DR5::GUS* expression in wild type was increased after 24 hours (data not shown) and 48 hours Cd^{2+} treatment (Figure 3.7b). In contrast, other metals tested including K^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, or Ca^{2+}, did not effect the *DR5::GUS* expression in wild type (data not shown).

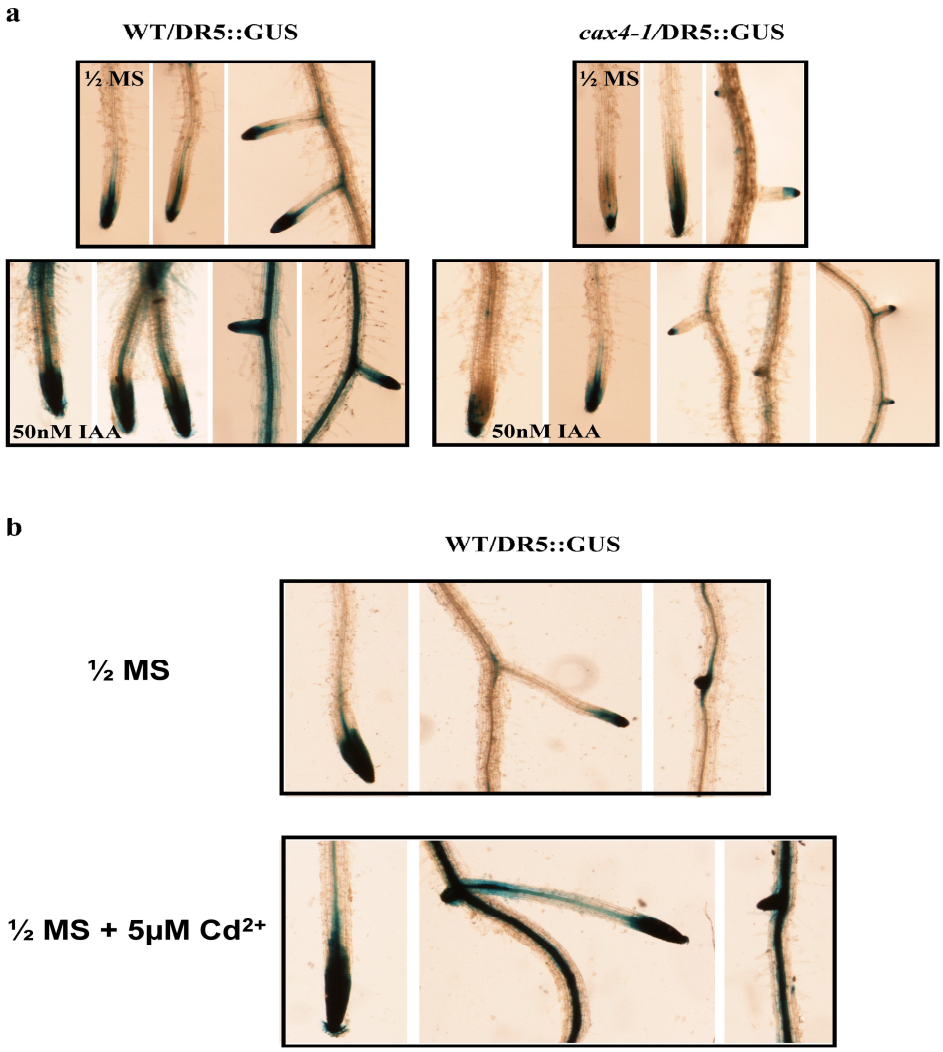


Figure 3.7 DR5::GUS assay for auxin responses and Cd^{2+} responses.

a. *DR5::GUS* assay for auxin responses in *cax4* allele. Top panel: wild type *DR5::GUS* seedlings and *cax4-1 DR5::GUS* seedlings were grown on ½ MS media for 12 days before stained for GUS activity. Bottom panel: wild type *DR5::GUS* seedlings and *cax4-1*/*DR5::GUS* seedlings were grown on ½ MS media for 10 days then transferred to ½ MS media containing 50 nM IAA for 2 days before stained for GUS activity. b. *DR5::GUS* assay for Cd^{2+} responses in wild-type plants. Top panel: wild type *DR5::GUS* seedlings were grown on ½ MS media for 12 days before stained for GUS activity. Bottom panel: wild type *DR5::GUS* seedlings were grown on ½ MS media for 10 days then transferred to ½ MS media containing 5 μM Cd^{2+} for 2 days before stained for GUS activity.

Discussion

The *Arabidopsis* genome contains six *CAX* transporter genes (Shigaki et al., 2006). To address the functional diversity and physiological role of these transporters, we have further characterized *CAX4*. In this study, we have demonstrated that *CAX4* is required for root growth and development under metal stress conditions. In particular, we have demonstrated that *CAX4* is specifically expressed in the primary root apex and lateral root primordia from early stages through post-emergence development under normal growth conditions (Figure 3.1). *CAX3* is also highly expressed in root tips and the root elongation zone but its expression is not root-specific (Cheng et al., 2005). Expression profiles of *CAXs* from rice (*Oryza sativa*) suggest that none of the five *OsCAXs* is exclusively expressed in roots (Kamiya et al., 2005; 2006). Although expression of *CAX4* is relatively low, it is modulated by environmental conditions. The *CAX4::GUS* signal was highly induced by Ni^{2+} and Mn^{2+} in the root apex and throughout the whole lateral roots (Figure 3.1). Contrary to *CAX1* and *CAX3*, whose expressions are up-regulated by exogenous Ca^{2+} (Hirschi, 1999; Shigaki and Hirschi, 2000), *CAX4::GUS* signal was only increased under Ca^{2+} limiting conditions (Figure 3.1). The induction of *CAX4* expression by Ca^{2+} depletion conditions is puzzling considering its putative vacuolar cation/H^+ functions in excess cation tolerance. This might be an indirect Ca^{2+} effect, such as when Ca^{2+} is depleted, the accumulation of other cations in the roots may be increased and this imbalance up-regulates *CAX4* expression. For example, root Ca^{2+} channels have good permeability for other cations, such as Mn^{2+} (White, 1998), and when Ca^{2+} is limiting, it might lead to the accumulation

of higher concentrations of other cations. Alternatively, *CAX4* may be induced as a response to low Ca^{2+} conditions in order to maintain a pool of Ca^{2+} in the vacuole. In sum, the expression data suggest that CAX4 functions in root growth and adaptation.

Among the CAXs, the root expression pattern of *CAX4* appears to be unique; however, when expressed at high levels in plants, its biochemical properties resembled other CAXs. Expression of *35S*::*CAX4* could partially suppress the *cax1* defect in vacuolar Ca^{2+}/H^{+} transport (Figure 3.4). Previous studies using yeast found that CAX4 could only transport Ca^{2+} if the N-terminus was modified (Cheng et al., 2002). This result therefore suggests that when expressed in *Arabidopsis*, CAX4 can be activated without requiring artificial deregulation. Previous work in both yeast and plant expression systems suggests that CAX4 may modulate both vacuolar Ca^{2+} and metal levels, including Cd^{2+}, depending on the environmental conditions (Park et al., 2005; Korenkov et al., 2007a, 2007b). Given the similar ionic radius of Cd^{2+} and Ca^{2+}, it is not surprising that CAX4 can transport both Cd^{2+} and Ca^{2+}. Evidence for a role of CAX4 in Ca^{2+} homeostasis is also suggested by the ion sensitivity phenotypes of tobacco and *Arabidopsis* plants expressing *35S*::*CAX4* (Figure 3.5 and 3.6). These phenotypes are similar to those of *sCAX1*-expressing tobacco and tomato plants (Hirschi, 1999; Park et al., 2005). Ectopic expression of *sCAX1* in tobacco causes sensitivity to Mg^{2+}, K^{+}, and Na^{+} stresses, which are associated with Ca^{2+} deficiency (Hirschi, 1999). We speculate that this is caused by Ca^{2+} deficiencies that arise from over-accumulation of Ca^{2+} into the vacuole. Consistent with this explanation, the addition of Ca^{2+} in the medium restored the normal growth of *CAX4*-expressing tobacco and *Arabidopsis* lines (Figure 3.5).

While *CAX4* expression caused numerous ion sensitivities, *CAX4*-expressing tobacco plants are also more tolerant to Cd^{2+} and Mn^{2+} (Korenkov et al., 2007a). The increased Cd^{2+}/H^{+} antiport activity and Cd^{2+} tolerance in *CAX4*-expressing tobacco and yeast (Cheng et al. 2002; Korenkov et al., 2007a) suggests that CAX4 is a cation transporter which has a high affinity for both Cd^{2+} and Ca^{2+}. We postulate that at the root apex, CAX4 is important for the regulation of Cd^{2+} and Ca^{2+} levels. It is likely, however, that the predominant substrate for CAX4 under normal growth conditions is Ca^{2+}.

The *CAX4* silencing experiments indicate that CAX4 functions in root growth and development during heavy metal stress conditions. The *cax4-1* and *CAX4* RNAi lines displayed altered root architectures only when exposed to Cd^{2+} or Mn^{2+} stress (Figure 3.3). Such Cd^{2+} sensitivity is also found in other Cd^{2+} transport mutants. For example, a knockout of *ATM3*, which encodes an ATP-binding cassette metal transporter, is more sensitive to Cd^{2+} than wild type plants (Kim et al., 2006). Likewise, knockout of the vacuolar-localized P_{1B}-type ATPase AtHMA3 causes Cd^{2+} sensitivity (Morel et al., 2008). These metal sensitivity root phenotypes of *cax4* are distinct from other *cax* lines. *Arabidopsis* lines deleted for *CAX3*, which is also highly expressed in roots, are sensitive to salt stress, low pH, and abscisic acid, but not Cd^{2+} (Zhao et al., 2008). These observations suggest that each CAX transporter may be involved in responding to specific environmental stress conditions. The *cax4* mutants root phenotypes are consistent with its biochemical functions as a Ca^{2+} and Cd^{2+} transporter and highlight its importance in root growth and development through mediating metal ion sequestration.

The results presented here support the idea that CAX4 cation/H^+ antiport activity is required for the fidelity of auxin-mediated root growth and development. The *CAX4* mutants exhibited altered responses to exogenous auxin (Figure 3.6) but no other hormones perturbed *cax4* mutant growth. A reduction in IAA content in the root tip in the *cax4* mutants, as indicated by reduced *DR5::GUS* expression (Figure 3.7a), would lead to loss of primary root meristem function and inhibition of root growth. A similar phenotype has been seen in plants over-expressing an AGC-type kinase PID (Friml et al., 2004). Previous studies have indicated a link between cytosolic Ca^{2+} signaling and auxin-regulated plant development via PID and other AGC kinases (Benjamins et al., 2003; Robert and Offringa, 2008). Ca^{2+} regulates these kinases through interaction with Ca^{2+} binding proteins and the kinase in turn regulates the PIN auxin efflux carrier. Thus it is possible that increased cytosolic Ca^{2+} levels that may occur in some root cells lacking *CAX4* (due to reduced transport into the vacuole) could cause altered auxin efflux, impaired root auxin gradients, and altered root development. In contrast, the auxin sensitivity phenotype could be due to altered homeostasis of metals such as Cd^{2+} and Mn^{2+} in cells lacking *CAX4*. Metal homeostasis plays a role in auxin responses because it is important for some aspects of IAA metabolism (Tam et al., 2000; Walz et al., 2002; Magidin et al., 2003; Rampey et al., 2006). For example, amidohydrolases that cleave IAA-amino acid conjugates require metal cofactors for activity, and metal homeostasis regulators are involved in this process (Lasswell et al. 2000; Magidin et al., 2003; Rampey et al., 2006). Our observations suggest a link between Cd^{2+} homeostasis and auxin responses. Previous studies have indicated links between auxin homeostasis

and Cd^{2+} stress (Hagen and Guilfoyle, 1985; Hagen et al., 1988; Rajkumar et al., 2005; Ganesan, 2008). It is possible that *cax4* lines that are unable to tolerate Cd^{2+} stress are also uncoupling auxin production and/or metabolism in roots. Further studies are required to determine the relationship between metal homeostasis, auxin sensitivity, and CAX4 function. Additionally, the relationship between specific CAX transporters and auxin signaling requires further studies. For example, roots of *cax1* lines are slightly more tolerant to auxin (Cheng et al., 2003) whereas *cax4* lines are more sensitive. This may be due to differences in expression between *CAX1* and *CAX4*. Ca^{2+} imaging studies and measurements of auxin transport in each *CAX* mutant will be useful in dissecting the specific roles of each transporter in auxin responses.

CHAPTER IV

AN ENDOMEMBRANE Ca^{2+}/H^+ ANTIPORTER IS REQUIRED FOR ZEBRAFISH (*Danio rerio*) NEURAL CREST DEVELOPMENT

Introduction

Ca^{2+} acts as ubiquitous second messengers in the signal transduction pathway to regulate many physiological processes, including embryogenesis and many aspects of development (Ashworth et al., 2001; Blader and Strahle., 2000; Creton, 2004). Ca^{2+} concentrations are maintained by transmembrane Ca^{2+} fluxes that are modulated by Ca^{2+} transporters both on the plasma membrane and on endomembranes. Analysis of the growing volume of genomic sequence data from a variety of species suggests many as-yet-uncharacterized proteins may contribute to transport of Ca^{2+} during growth and development.

In yeast and plant cells, CAtion/H^+ eXchanger (CAX) proteins have been identified as playing a role in the restoration of low cytosolic Ca^{2+} levels after Ca^{2+} signaling events (Cunningham and Fink, 1995; Cheng et al., 2003; Zhao et al., 2008). CAX proteins use a gradient of H^+ generated by proton pumps to export Ca^{2+} or other cation out of the cytosol (Kamiya and Maeshima, 2004). There are 186 CAX open reading frames (ORF) sequences deposited in the public databases and the phylogenetic analysis demonstrates that the CAX family consists of three major types of CAXs: Type I, II , and III (Shigaki et al., 2006). Several Type I and Type III CAXs have been well characterized including yeast Vcx1 and *Escherichia coli* ChaA (Cunningham and Fink,

1995; Ivey et al., 1993; Ohyama et al., 1994; Naseem et al., 2008). However, there is a dearth of information available regarding Type II CAXs, and still less on their role in growth and development.

Previously characterized CAXs are all from plants, bacteria, and fungi and speculation has been that H^+-dependent Ca^{2+} transporters are not found in animal cells (Cai and Lytton, 2004). Genome analysis suggests that Type II CAXs are present in a broad range of organisms (Shigaki et al., 2006). In fact, Type II CAXs are present in some animal species, including *Xenopus*, the sea unchin and fish species. When compared to Type I and III CAXs, Type II CAXs have a unique N-terminal secondary structure and two or more additional transmembrane (TM) domains at the N-terminus. To date, the only Type II CAX that has been functionally characterized is the yeast (*S. cerevisiae*) Vnx1 (Cagnac et al., 2007). The transport data suggests Vnx1 helps regulate cytosolic cation concentrations and perhaps pH levels, but the biological role of Type II CAXs remains speculative.

Zebrafish (*Danio rerio*) has emerged as a powerful model for dissecting the relationship between Ca^{2+} signaling and development (Berridge et al., 2000; Ashworth et al, 2007; Lamason et al., 2005; Ginger et al., 2008). Ca^{2+} signaling plays a crucial role ranging from initiation of fertilization through embryonic patterning and oragnogenesis (Berridge et al., 2000; Webb and Miller, 2006; Ashworth et al, 2007). Ca^{2+} signaling mediates the induction and establishment of the highly migratory pluripotent neural crest cells that give rise to a variety of differentiated cell types, such as cranial cartilage, neurons, and pigment cells (Bouvard et al., 1998; Price et al., 2003; Jin et al., 2005), The

Ca^{2+} signaling that directs crest cell migrations and influences differentiation have been extensively studied, but the transporters involved in the Ca^{2+} transport and distribution to regulate the intracellular Ca^{2+} concentration have been less well understood. zebrafish CAX homologs may serve as valuable tools to dissect the function of animal CAX transporters in growth and development.

Here we report the cloning and characterization of zebrafish *cax1,* a Type II CAX. Using yeast as an experimental tool, we examined the subcelluar localization and biochemical function of this animal CAX. Our genetic analysis in zebrafish suggests that Cax1 functions as a Ca^{2+} transporter that is required for neural crest development.

Materials and Methods

Phylogenetic analysis

Protein BLAST searches were performed in August, 2005, using the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/BLAST/) on the nonredundant protein database. As the reference sequences for BLAST, *Saccharomyces cerevisiae* Vnx1 was used. We identified 51 sequences that shows a characteristic Type II CAX secondary structure and these were used for phylogenetic analyses.

Multiple sequence alignments (Supplementary Material) were performed using ClustalW, available on the European Bioinformatics Institute Web site (http://www.ebi.ac.uk/clustalw/), with additional manual adjustments. Only the homologous regions were used for the multiple sequence alignments. The phylogenetic

tree on the data sets was constructed using Protdist and Fitch programs in the phylogenetic suite Phylip (Felsenstein, 1996). The tree file was visualized by the program Treeview (Page, 1996).

Yeast strain and plasmid

The yeast (*Saccharomyces cerevisiae*) *vnx1Δ* strain was generated by replacing the *YNL321W* ORF with the kanMX6 gene by PCR-based gene deletion method (Longtine et al., 1998) using the following primers: YNL321w-F1 5' AAGTGAAATAACTGCTAGCTAGAAGAGCGGTAAGCAGCACGGATCCCCGGG TTAATTAA-3' and YNL321w-R1 5'- AAAATTGGTAGGTATCCAGGTGAAAAGCGGGGAC AGTTGCGAATCGCTCGTTTAAAC-3'. Genomic DNA from Geneticin-resistant strains was isolated and insertion of the disruption cassette into the correct locus was verified by PCR. The *vnx1Δ pmr1Δ* strain was generated by crossing *vnx1Δ to pmr1Δ* (Cunningham and Fink, 1996). The *vnx1Δ* and *vnx1Δ pmr1Δ* strains were transformed and grown as described previously by Shigaki et al. (2003). The *cax1* and HA-Cax1 clones were subcloned into the yeast expression vector piHGpd (Nathan *et al.*, 1999) as described in previous studies (Pittman and Hirschi, 2001; Pittman *et al.*, 2002*a*).

Yeast culture conditions, sample processing, and ICP-AES analysis

Yeast culture conditions and sample processing were modified from a previous study (Eide *et al.*, 2005). Yeast cultures were inoculated in 5 ml yeast–peptone–dextrose (YPD) and 1/100 vol. of 100x mineral supplement stock (Eide *et al.*, 2005) with additional 10 mM $CaCl_2$. The cells were grown at 30 °C to stationary phase. A 2.5 ml

sample of each culture was collected by vacuum filtration using isopore membrane filters (1.2 µm pore size) (Fisher Scientific, PA, USA). Cells were washed three times with 1 ml of 1 µM ethylenediaminetetraacetic acid disodium salt solution, pH 8.0, by vacuum filtration followed by three washes with 1 ml of distilled, deionized H_2O. The filters were dried at 70 °C in an oven for 48 h before inductively coupled plasma atomic emission spectroscopy (ICP-AES) analysis, performed as previously described (Lahner *et al.*, 2003).

Membrane fractionation and Western analysis

Microsomal membranes were prepared from yeast expressing HA-Cax1 as described by Pittman and Hirschi, (2001). Immunoblots were performed and the HA epitope was detected essentially as described previously (Hirschi et al., 1998; Pittman and Hirschi, 2001).

$^{45}Ca^{2+}$ transport assays

Calcium transport in sucrose gradient fractions was assayed as described by Sorin et al. (1997).

Fish strains

The wild-type strain was derived from the AB line (Eugene, OR). Embryos were developed in an incubator at 28.5°C in fish water containing 0.008% Instant Ocean salts.

Morpholino injections

Morpholino oligomers obtained from Gene Tools Inc. were diluted and injected as previously described (Nasevicius and Ekker, 2000✦; Phillips et al., 2001✦). A total of

1-5 ng MO was injected per embryo. At least 25 specimens were examined for each experiment.

To knockdown *cax1,* translation-blocking morpholinos and splice-blocking morpholinos were generated as follows: translational blocking MO: 5' ACATCGTGGGTTTGATTTGAGCCAT 3'; splice blocker exon1/2 (splice donor site): 5' AGATGATTATAATACTGACCTGAGC 3'; splice blocker exon2/3 (splice acceptor site): 5' CTTAAATACTCACTGTGGGCAGAGA 3'; Unless stated otherwise, *pax8* morphants were injected with 2.5 ng each of variant 1 MO and variant 2/3 MO to maximally disrupt *cax1* function.

In situ *hybridization*

In situ hybridization was carried out as described in Phillips et al. (Phillips et al., 2001). Antisense riboprobes were transcribed from plasmids containing *cax1*.

Alcian blue staining

Staining with Alcian blue was performed as described by Solomon et al. (2003).

Mis-expression of Cax1

Full length *cax1* was cloned into pCS2 expression vector. RNA was synthesized in vitro and 200ng RNA or 300ng was injected into the yolk of cleaving embryos at the one- cell stage, or was co-injected with *cax1* MO. To misexpress Cax1 under the control of the cytomegalovirus promoter, 30-60 pg plasmid DNA was injected into one-cell embryos, or was co-injected with *cax1* MO.

Immuno-staining

Antibody staining was performed as described by Phillips et al. (2006). Embryos were incubated with primary monoclonal antibodies Pax7 (1:150) purchased from the Developmental Studies Hybridoma Bank. Embryos were washed and incubated with Alexa 488 goat anti mouse IgG (Molecular Probes A-11001, diluted 1:50).

Results

Identification of zebrafish cax1

To study the function of animal Type II CAXs, we set out to clone zebrafish *cax1* by a PCR based approach. Microarray data suggests that the highest level of zebrafish *cax1* expression in zebrafish embryos is at 24 hpf (hours post-fertilization). Therefore, total RNA was extracted from zebrafish 24 hpf embryos and used to amplify the *cax1* full length cDNA by using gene specific primers. The *cax1* cDNA consists of 2,295 nucleotides, which code for a polypeptide containing 764 amino acids (accession no. DQ631793, Fig. 4.1). The deduced protein was aligned with previously identified yeast Type II CAX, Vnx1, and other animal Type II CAXs (Fig. 4.1). zebrafish Cax1 was only 28% identical (33% similarity) to Vnx1 and exhibited much higher identity to other animal Type II CAXs. It is 66% identical (77% similarity) to zebrafish Cax2, 62% identical (76% similarity) to frog type II CAX, and 64% identical (77% similarity) to fugu fish type II CAX (Fig. 4.1).

The phylogenetic analysis of all 51 Type II CAX sequences that were available from public databases (www.ncbi.nlm.nih.gov) indentified several subgroups (Fig. 4.1).

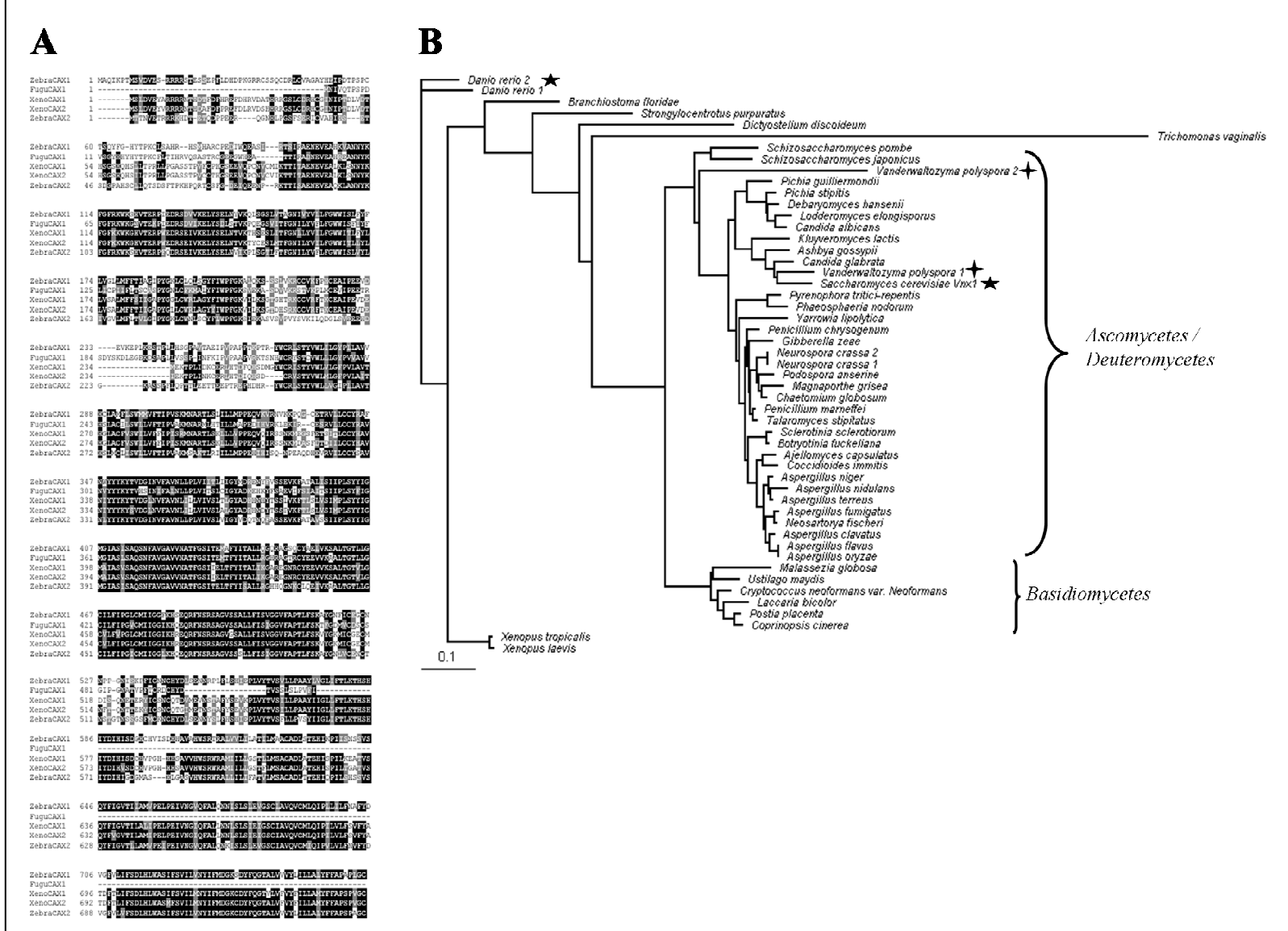


Figure 4.1 Phylogenetic analysis of Type II CAXs.

A Alignment of deduced amino acid sequences of polypeptides encoded by zebrafish *cax1* and *cax2*, Xenopus *cax1* and *cax2,* and Fugu *cax1*. Consensus amino acid residues are boxed in black (identical) or gray (similar). B. Phylogenetic tree made from 51 full-length Type II CAXs in the public database. The tree branches are labeled with the species from which each CAX was derived. When there are multiple CAXs from the same species, they are distinguished by a number after the specific epithet. The zebrafish Cax1, and yeast Vnx1 are marked with "★" and two *Vandervaltozyma polyspora* CAXs are marked with "✦".

Type II CAXs from Ascomycetes and Deuteromycetes formed one large group and Type II CAXs from Basidiomycetes formed another group (Fig. 4.1). Fish and Xenopus Type II CAXs were distant from these two groups. Most fungal species only contained one copy of Type II CAXs. However, a yeast species *Vandervaltozyma polyspora* contained two Type II CAXs that were not clustered together (Fig. 4.1).

Subcellular localization of Cax1 in yeast

To initially test the properties of zebrafish Cax1, we expressed the open reading frame in various yeast (*Saccharomyces cerevisiae)* strains. To identify the cellular localization of Cax1 in yeast, an epitope-tagged variant was generated by the fusion of triple copy of HA (hemagglutinin) to the N terminus (HA-Cax1). An N-terminal HA epitope tag has previously been used to identify the cellular location of other CAXs in yeast (Cunningham and Fink, 1999; Pittman and Hirschi, 2001; Shigaki et al., 2001). In yeast cells, HA-Cax1 was expressed under the control of the constitutive glyceraldehydes phosphate dehydrogenase promoter (GPD; Nathan et al., 1999) and localized to endomembranes but not to the vacuolar membranes (Fig. 4.2).

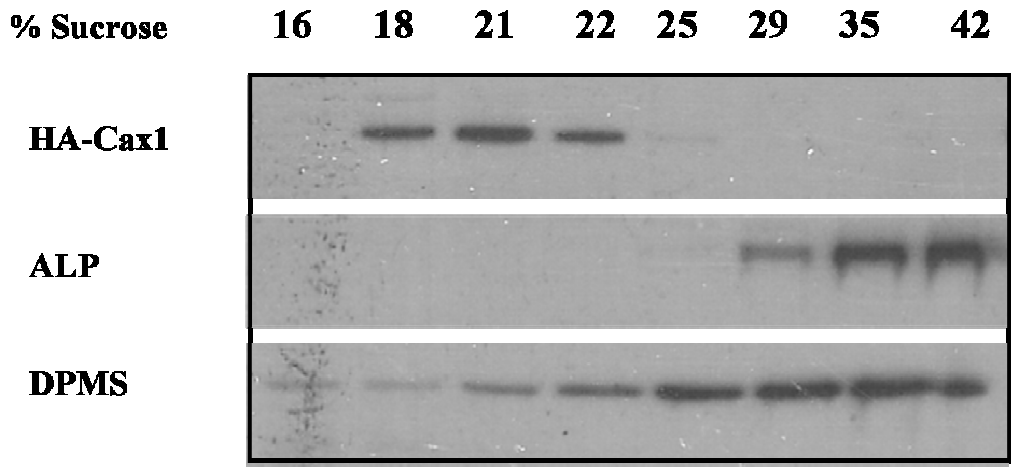


Figure 4.2 Subcellular fractionation of HA-tagged Cax1.

The W303 yeast strain was transformed with HA-Cax1 grown in standard yeast extract-peptone-dextrose medium and membranes were fractionated on a sucrose gradient (15–48%, w/w). Protein extracts (10 mg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Proteins were immunodetected with an immune serum specific for hemagglutinin, yeast vacuolar alkaline phosphatase (ALP) and the yeast endoplasmic reticulum protein dolichol phosphate mannose synthase (DPMS).

Functional characterization of cax1 in yeast mutant cells

To date the majority of functionally characterized CAXs have some role in calcium transport (Hirschi et al., 1999; Cheng et al., 2003). We were interested in further investigating the role of Vnx1 in calcium homeostasis as a means to dissect zebrafish Cax1 function. Recent technologies make it possible to measure the sum total of all the mineral nutrients and trace elements in the cell, termed the ionome (Lahner et al., 2003). When putative transporters are perturbed in yeast, the alterations in the ionome profile have been used to infer function (Eide et al., 2005; Cheng et al., 2005; Mei et al., 2007). Under normal conditions, the ionome of *vnx1Δ* is similar to wild type (Eide et al., 2005); however, when grown in elevated levels of calcium, *vnx1Δ* cells accumulated 64% less calcium than controls (Fig. 4.3). Other metal levels were similar among all the yeast cells tested (data not shown). These observations suggest that under particular environmental conditions Vnx1 may transport Ca^{2+} (Fig. 4.3). Endomembrane Vcx1 independent Ca^{2+}/H^{+} transport activity can be measured in yeast cells deficient in Pmr1, a Golgi Ca^{2+}-ATPase (Marchi et al., 1999). In order to determine if this Ca^{2+}/H^{+} transport activity is mediated by Vnx1, we made a *vnx1Δ pmr1Δ* double mutant and examined Ca^{2+} transport activity. In *vnx1Δ pmr1Δ* cells, the endomembrane Ca^{2+}/H^{+} transport activity that was detected in *pmr1Δ* cells was absent (Fig. 4.4)

To test the function of zebrafish Cax1, we expressed the putative animal transporter in *vnx1Δ* and *vnx1Δ pmr1Δ* cells. Ionome analysis demonstrated calcium accumulation increased two-fold in Cax1-expressing *vnx1Δ* cells compared with controls (Fig. 4.3). We also examined the ionome profile of HA-Cax1-expressing *vnx1Δ* cells and

they accumulated calcium comparable to Cax1-exprssing *vnx1Δ* cells suggesting the tagged protein remained functional (Fig. 4.3). Directly measuring cax1 transport properties confirmed the ionome data. When expressed in *vnx1Δ pmr1Δ* cells, Cax1 restored the endomembrane Ca^{2+}/H^{+} transport activity and the transport properties were indistinguishable from *pmr1Δ* cells (Fig. 4.4).

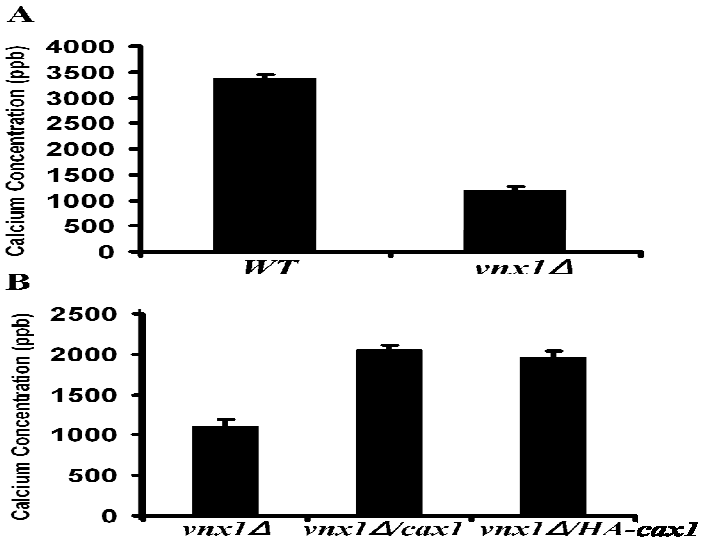


Figure 4.3 Concentration of total calcium in wild type, *vnx1Δ, vector-, cax1-,* and *HA-cax1* expressing *vnx1Δ* yeast cells.

A. Concentration of total calcium in wild type and *vnx1Δ* yeast cells grow in yeast-peptone-dextrose (YPD) medium with mineral supplements and additional 10 mM $CaCl_2$. B. Vector-, *cax1-*, and *HA-cax1* expressing *vnx1Δ* yeast cells grow in yeast-peptone-dextrose (YPD) medium with mineral supplements and additional 10 mM $CaCl_2$. Data represent the means (±SE) from three independent analyses.

cax1 is expressed in zebrafish neural crest cells

To determine the developmental role of zebrafish *cax1* in zebrafish, we first examined the expression pattern of *cax1* by using whole mount RNA *in situ* hybridization. Two probes were used, one containing 1.6 kb *cax1* N-terminal coding sequence, and a second specific to *cax1*containing a 0.3 kb *cax1* N-terminal sequence. Both probes gave similar results and for subsequent studies the 1.6 kb probe was used (Fig. 4.5). At 24 and 48 hpf, *cax1* was predominantly detected in neural crest cells (Fig. 4.5). To test if *cax1* was exclusively observed in neural crest, we examined *cax1* expression in a double morphants of *tfap2a/tfap2c* that is neural crest deficient (Li and Cornell, 2007). In these lines, *cax* expression could no longer be detected at 24 hpf (Fig. 4.5).

Cax1 is required for normal neural crest development

We investigated the effects of zebrafish Cax1 disruption by antisense morpholino knockdown. We designed three different morpholinos, one to block translation of *cax1* and two to block pre-mRNA splicing. The *cax1* translation blocker (TB) and *cax1* splice blocker 2 (SB2) both affected neural crest development although to differing degrees of severity (Fig. 4.5, and 4.6). However, the *cax1* splice blocker 1 (SB1) was ineffective and did not display a significant phenotype. RT-PCR confirms that SB2 was able to knock down *cax1* while SB1 was not effective at knocking down the targeted mRNA (data not shown). p53 was co-injected with *Cax1* SB2 in all experiments to reduce non-specific cell death due to morpholino off-targeting seen in *cax1* SB2 morphants, p53 was

co-injected with *Cax1* SB2 for all experiments (Robu et al., 2007). In agreement with the expression of *Cax1*, the phenotype of *cax1* morphants (MOs) were specific to neural

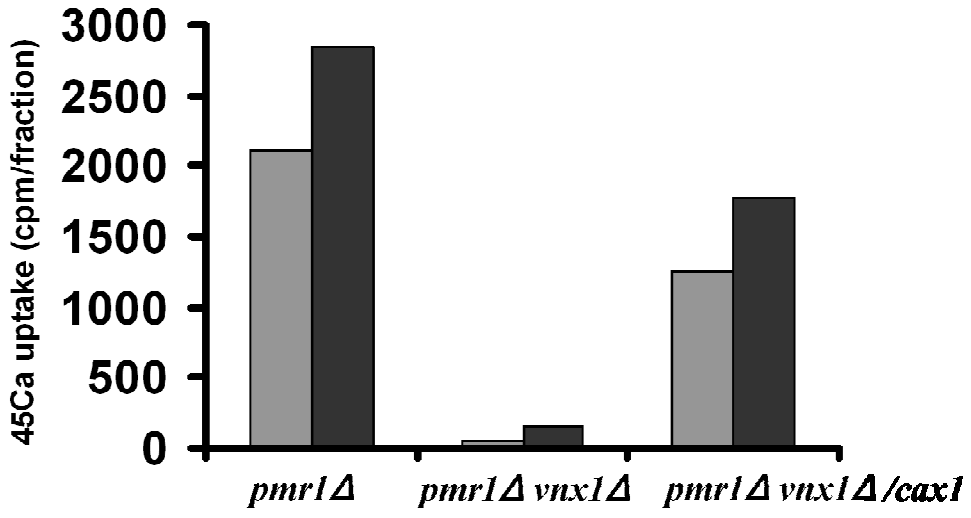


Figure 4. 4 Ca^{2+}/H^{+} antiport activity in *pmr1Δ, vnx1Δ pmr1Δ, and vnx1Δ pmr1Δ/cax1* yeast.

Ca^{2+}/H^{+} antiport activity was determined in two endomembrane-enriched but not vacuolar membrane-enriched fractions of sucrose density gradient separations of yeast lysates. Data are representatives of two independent experiments.

crest development. Neural crest cells give rise to a variety of different cell types and contribute to a number of body structures including elements of the jaw and the characteristic horizontal-stripe pigment pattern. At 30 hpf, the pigmentation of *cax1* morphants was significantly reduced however, by 48h this phenotype was less evident (Fig. 4.6). Alcian blue staining of jaw cartilage in *cax1* morphants at 72 hpf indicated

that disruption of *cax1* function caused defects in jaw structure (Fig. 4.6). Disruption of *cax1* function also caused defects in jaw development as seen with 72 hpf alcian blue staining of jaw cartilage. The gill arches, dorsal palate and mandibular cartilages were most severely affected in *cax1* SB2 morphants and to a lesser degree in *cax1* TB morphants. These jaw structures were either absent or reduced in size in *cax1* morphants. We also examined expression of the neural crest marker, paired box protein Pax7, in *cax1* morphants (Lacosta et al., 2007). At 24 hpf, there were fewer Pax7-expressing neural crest cells adjacent to hindbrain level (Rhombomere 1-3) in *cax1* morphants (5 ± 7) compared to controls (11 ± 3) (Fig. 4.6 and Table 4.1). This is not a developmental delay as it can also be seen at 30 hpf (Fig. 4.6).

Misexpression of cax1

The pax7 phenotype was rescued by coinjecting cax1 mRNA resistant to MO knock down or coinjection of cax1 plasmid DNA. However, injecting 300pg of *cax1* mRNA without MOs also caused a reduction of Pax7 expressing neural crest cells (data not shown). The defects in jaw cartilage in *cax1* morphants could not be rescued using

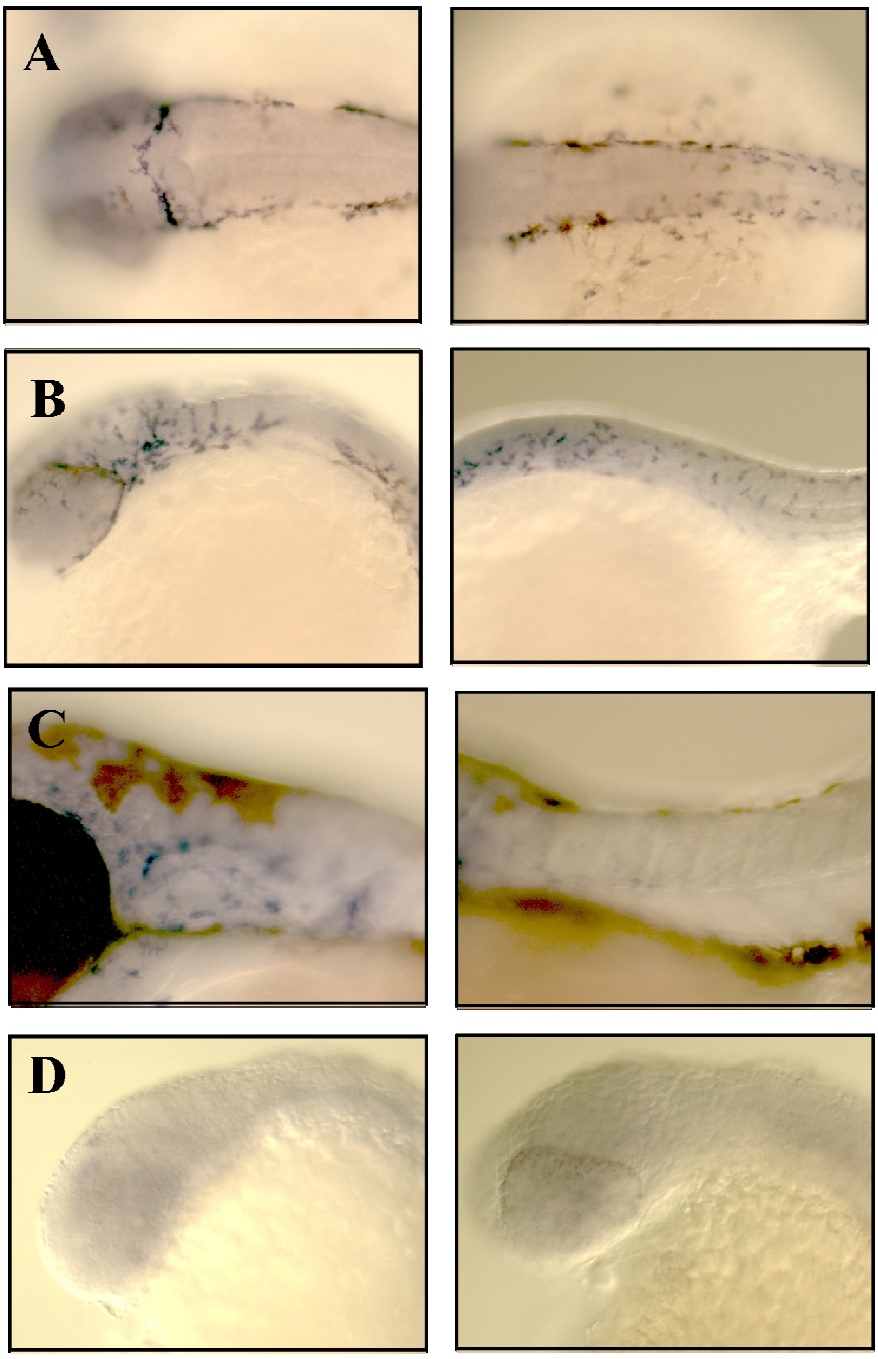


Figure 4. 5 Expression of *cax1*.

Panel A show dorsal views. Panel B, C, and D show lateral views. A and B. Wild type embryos at 24 hpf showed *cax1* expression in neural crest cells. C. Wild type embryos at 48 hpf showed low *cax1* expression in neural crest cells. D. Embryos of *tfap2a/tfap2c* morphants at 24 hpf showed no *cax1* expression.

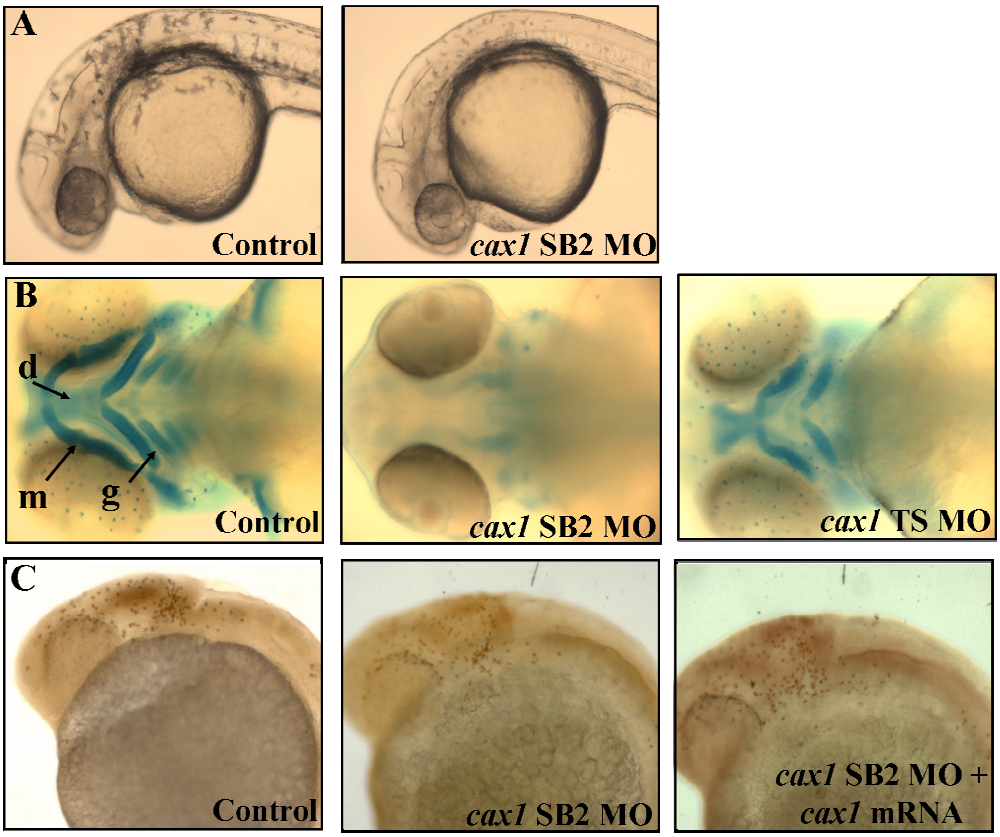


Figure 4. 6 Impaired neural crest development in *cax1* morphants.

A. The live image of 30 hpf embryos showed the slightly smaller head and reduced pigmentation in *cax1* morphants B. Imparied neural crest development in *cax1* morphants. Alcian blue cartilage staining at 72 hpf in zebrafish embryos. Abbreviations: d, dorsal palate; g, gill arches; m, mandibular cartilage. Images show ventral views with anterior to the left. C. Anti-Pax7 staining at 24 hpf in embryos of a control, *cax1* morphants, and *cax1* morphants coinjected with *cax1* mRNA or DNA as indicated in the figure.

Table 4.1	**No. of Pax7 expressing cells**
Control	8.8 ± 4.35 (n=15)
cax1 MO	4.4 ± 2.9 (n=18) *P<0.0001
cax1 MO + cax1 RNA	10.25 ± 3.7 (n=16) *P=0.16 ‡P<0.0001
	No. of Pax7 expressing cells
Control	16.3 ± 4.7 (n=12)
cax1 MO	9.14 ± 5.3 (n= 18) *P<0.0001
cax1 MO + cax1 DNA	14 ± 4.1 (n=16) *P=0.05 ‡P=0.0002

Discussion

A number of CAXs from plants and microorganisms have been functionally characterized; however, this study marks the initial characterization of an animal CAX.

The phylogenetic analysis suggests that the animal Type II CAXs are not closely related to fungal CAXs. However, two zebrafish CAXs are very similar to each other and zebrafish *Cax1* is located on chromosome 4 and zebrafish *Cax2* is located on chromosome 25 suggesting they might be paralogous. Based on phylogeny, some species appear to have two distinct Type II CAXs, inferring diversity of function. The phylogenetic distance between Vnx1 and zebrafish Cax1 implies that while they may have some similar biological functions (see below) the role of zebrafish cax1 is distinct from yeast Vnx1. While Type II CAXs are present in aquatic animal species, including fish species, the sea urchin, and Xenopus, CAXs are absent in terrestrial species. We speculate that the presence of CAXs in aquatic species is necessary for them to adapt to different water environments.

Biochemically we have demonstrated in this study that Cax1 facilitates yeast endomembrane Ca^{2+} uptake. While these localization studies are not directly applicable to zebrafish endomembranes, they facilitate the initial biochemical characterization of cax1. When expressed in yeast endomembranes, cax1 can suppress yeast mutants defective in Ca^{+} uptake (Fig. 4.2 and 4.4). Additionally, *cax1* expressing yeast cells demonstrated increased calcium accumulation compared to controls (Fig. 4.3). Several endomembrane cation/H^{+} transporter have been characterized in plants and mammalian cells (Ettinger et al., 1999; Venema et al., 2003; Sudhir et al., 1998; Numata and Orlowski, 2001). For example, human *NHE7* is a Na^{+}/H^{+} exchanger and localizes to the trans-Golgi network; the plant K^{+}/H^{+} exchanger, LeNHX2, co-localizes with prevacuolar and Golgi markers in both yeast and plants. Emerging evidence suggests the luminal acidification of organelles may be the driving force for cation/H^{+} antiporter in both plants and animals (Shigeri et al., 2004; Dunant et al., 2009). Future studies will be directed toward more precisely comparing and contrasting cax1 transport with other Type II CAXs, most notably Vnx1.

Our findings suggest Cax1 is required for neural crest development but may also have a role in pigmentation. Our expression studies are in an agreement with microarray data which suggest transitory *cax1* expression is zebrafish embryos at 24 hpf (Fig. 4.5; Ming et al., 2008). The cax1 MO reduction in Pax7 predominately in the region adjacent to Rhombomere 1-3 also suggests the function of this transporter is spatially restricted within the neural crest. The defects in jaw structure and pigmentation are consistent with impaired neural crest differentiation and survival (Fig. 4.6; Phillips et al., 2006). Ectopic

expression of *cax1* rescued the Pax7 phenotype but both the pigmentation and jaw defects persisted. Our working model to explain these observations is that any alteration in Cax1 expression alters pigmentation and jaw development. To our knowledge, this is the first calcium transporter shown to function in neural crest development; however, previous studies have firmly established the role of calcium transporters in both zebrafish and human pigmentation (Lamason et al., 2005; Ginger et al., 2008)

Our results do not define the kinetics of Cax1-mediated Ca^{2+} transport and direct evidence is lacking that Cax1 is involved in Ca^{2+} distribution within the neural crest. However, it is evident from the morphant phenotypes that Cax1 plays a crucial role in zebrafish neural crest development. Although it is not clear whether other animal CAXs have similar functions, our results highlight the importance of CAXs in zebrafish embryo growth and development.

CHAPTER V

SUMMARY

Using a combination of approaches, I attempt to characterize the expression and physiological function of cation/H^+ transporters. The first project was aimed at using yeast and transgenic tobacco plants to understand the regulation of *Arabidopsis* CAX1 in *planta*. The second project was using molecular and genetics approaches to characterize the physiological role of *Arabidopsis* CAX4 in roots. The results showed that CAX4 is expressed in roots and is required in root development under heave metal stress. The third project was to characterize the first animal CAX, zebrafish Cax1. The results showed that zebrafish Cax1 is expressed in neural crest cells and is important for neural crest development. Further experiments and analysis of other animal CAXs will be need to fully understand the role of these genes play in animal growth and development.

REFERENCES

Aiello, D.P., Fu, L., Miseta, A., Sipos, K., and Bedwell, D.M. (2004). The Ca^{2+} homeostasis defects in a pgm2Delta strain of *Saccharomyces cerevisiae* are caused by excessive vacuolar Ca^{2+} uptake mediated by the Ca^{2+}-ATPase Pmc1p. J Biol Chem **279,** 38495-38502.

Anraku, Y., Ohya, Y., and Iida, H. (1991). Cell cycle control by calcium and calmodulin in *Saccharomyces cerevisiae*. Biochim Biophys Acta **1093,** 169-177.

Ashworth, R., Zimprich, F., and Bolsover, S.R. (2001). Buffering intracellular calcium disrupts motoneuron development in intact zebrafish embryos. Brain Res Dev Brain Res **129,** 169-179.

Ashworth, R., and Brennan, C. (2005). Use of transgenic zebrafish reporter lines to study calcium signalling in development. Brief Funct Genomic Proteomic **4,** 186-193.

Babu, Y.S., Bugg, C.E., and Cook, W.J. (1988). Structure of calmodulin refined at 2.2 A resolution. J Mol Biol **204,** 191-204.

Baekgaard, L., Fuglsang, A.T., and Palmgren, M.G. (2005). Regulation of plant plasma membrane H^+- and Ca^{2+}-ATPases by terminal domains. J Bioenerg Biomembr **37,** 369-374.

Batiza, A.F., Schulz, T., and Masson, P.H. (1996). Yeast respond to hypotonic shock with a calcium pulse. J Biol Chem **271,** 23357-23362.

Benjamins, R., Ampudia, C.S., Hooykaas, P.J., and Offringa, R. (2003). PINOID-mediated signaling involves calcium-binding proteins. Plant Physiol **132,** 1623-1630.

Berridge, M.J., Lipp, P., and Bootman, M.D. (2000). The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol **1,** 11-21.

Berridge, M.J. (2004). Calcium signal transduction and cellular control mechanisms. Biochim Biophys Acta **1742,** 3-7.

Bertl, A., and Slayman, C.L. (1990). Cation-selective channels in the vacuolar membrane of Saccharomyces: dependence on calcium, redox state, and voltage. Proc Natl Acad Sci U S A **87,** 7824-7828.

Bertl, A., and Slayman, C.L. (1992). Complex modulation of cation channels in the tonoplast and plasma membrane of *Saccharomyces cerevisiae*: single-channel studies. J Exp Biol **172,** 271-287.

Bertl, A., Gradmann, D., and Slayman, C.L. (1992). Calcium- and voltage-dependent ion channels in *Saccharomyces cerevisiae*. Philos Trans R Soc Lond B Biol Sci **338,** 63-72.

Birchwood, C.J., Saba, J.D., Dickson, R.C., and Cunningham, K.W. (2001). Calcium influx and signaling in yeast stimulated by intracellular sphingosine 1-phosphate accumulation. J Biol Chem **276,** 11712-11718.

Blader, P., and Strahle, U. (2000). Zebrafish developmental genetics and central nervous system development. Hum Mol Genet **9,** 945-951.

Blumwald, E., and Poole, R.J. (1986). Kinetics of Ca/H antiport in isolated tonoplast vesicles from storage tissue of beta vulgaris L. Plant Physiol **80,** 727-731.

Bonilla, M., and Cunningham, K.W. (2002). Calcium release and influx in yeast: TRPC and VGCC rule another kingdom. EMBO J. **21,** 2343-2353.

Bonilla, M., Nastase, K.K., and Cunningham, K.W. (2002). Essential role of calcineurin in response to endoplasmic reticulum stress. EMBO J **21,** 2343-2353.

Bonilla, M., and Cunningham, K.W. (2003). Mitogen-activated protein kinase stimulation of Ca^{2+} signaling is required for survival of endoplasmic reticulum stress in yeast. Mol Biol Cell **14,** 4296-4305.

Bonza, M.C., Luoni, L., and De Michelis, M.I. (2004). Functional expression in yeast of an N-deleted form of At-ACA8, a plasma membrane Ca^{2+}-ATPase of *Arabidopsis thaliana*, and characterization of a hyperactive mutant. Planta **218,** 814-823.

Botstein, D., and Fink, G.R. (1988). Yeast: an experimental organism for modern biology. Science **240,** 1439-1443.

Botstein, D., Chervitz, S.A., and Cherry, J.M. (1997). Yeast as a model organism. Science **277,** 1259-1260.

Bouvard, D., and Block, M.R. (1998). Calcium/calmodulin-dependent protein kinase II controls integrin alpha5beta1-mediated cell adhesion through the integrin cytoplasmic domain associated protein-1alpha. Biochem Biophys Res Commun **252,** 46-50.

Busch, W., and Saier, M.H., Jr. (2002). The transporter classification (TC) system, 2002. Crit Rev Biochem Mol Biol **37,** 287-337.

Cagnac, O., Leterrier, M., Yeager, M., and Blumwald, E. (2007). Identification and characterization of Vnx1p, a novel type of vacuolar monovalent cation/H^+ antiporter of *Saccharomyces cerevisiae*. J Biol Chem **282,** 24284-24293.

Cai, X., and Lytton, J. (2004). The cation/Ca^{2+} exchanger superfamily: phylogenetic analysis and structural implications. Mol Biol Evol **21,** 1692-1703.

Catterall, W.A. (2000). From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. Neuron **26,** 13-25.

Cheng, N.H., Pittman, J.K., Shigaki, T., and Hirschi, K.D. (2002). Characterization of CAX4, an *Arabidopsis* H^+/cation antiporter. Plant Physiol **128,** 1245-1254.

Cheng, N.H., Pittman, J.K., Barkla, B.J., Shigaki, T., and Hirschi, K.D. (2003). The *Arabidopsis* cax1 mutant exhibits impaired ion homeostasis, development, and hormonal responses and reveals interplay among vacuolar transporters. Plant Cell **15,** 347-364.

Cheng, N.H., Liu, J.Z., Nelson, R.S., and Hirschi, K.D. (2004). Characterization of CXIP4, a novel *Arabidopsis* protein that activates the H^+/Ca^{2+} antiporter, CAX1. FEBS Lett **559,** 99-106.

Cheng, N.H., Pittman, J.K., Zhu, J.K., and Hirschi, K.D. (2004). The protein kinase SOS2 activates the *Arabidopsis* H^+/Ca^{2+} antiporter CAX1 to integrate calcium transport and salt tolerance. J Biol Chem **279,** 2922-2926.

Cheng, N.H., Pittman, J.K., Shigaki, T., Lachmansingh, J., LeClere, S., Lahner, B., Salt, D.E., and Hirschi, K.D. (2005). Functional association of *Arabidopsis* CAX1 and CAX3 is required for normal growth and ion homeostasis. Plant Physiol **138,** 2048-2060.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J **16,** 735-743.

Crabtree, G.R. (2001). Calcium, calcineurin, and the control of transcription. J Biol Chem **276,** 2313-2316.

Creton, R. (2004). The calcium pump of the endoplasmic reticulum plays a role in midline signaling during early zebrafish development. Brain Res Dev Brain Res **151,** 33-41.

Cunningham, K.W., and Fink, G.R. (1994). Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking PMC1, a homolog of plasma membrane Ca^{2+} ATPases. J Cell Biol **124,** 351-363.

Cunningham, K.W., and Fink, G.R. (1994). Ca^{2+} transport in *Saccharomyces cerevisiae.* J Exp Biol **196,** 157-166.

Cunningham, K.W., and Fink, G.R. (1996). Calcineurin inhibits VCX1-dependent H^+/Ca^{2+} exchange and induces Ca^{2+} ATPases in *Saccharomyces cerevisiae*. Mol Cell Biol **16,** 2226-2237.

Curran, A.C., Hwang, I., Corbin, J., Martinez, S., Rayle, D., Sze, H., and Harper, J.F. (2000). Autoinhibition of a calmodulin-dependent calcium pump involves a structure in the stalk that connects the transmembrane domain to the ATPase catalytic domain. J Biol Chem **275,** 30301-30308.

Cyert, M.S. (2001). Genetic analysis of calmodulin and its targets in *Saccharomyces cerevisiae*. Annu Rev Genet **35,** 647-672.

Cyert, M.S. (2003). Calcineurin signaling in *Saccharomyces cerevisiae*: how yeast go crazy in response to stress. Biochem Biophys Res Commun **311,** 1143-1150.

Denis, V., and Cyert, M.S. (2002). Internal Ca^{2+} release in yeast is triggered by hypertonic shock and mediated by a TRP channel homologue. J Cell Biol **156,** 29-34.

Devasahayam, G., Ritz, D., Helliwell, S.B., Burke, D.J., and Sturgill, T.W. (2006). Pmr1, a Golgi Ca^{2+}/Mn^{2+}-ATPase, is a regulator of the target of rapamycin (TOR) signaling pathway in yeast. Proc Natl Acad Sci U S A **103,** 17840-17845.

Devasahayam, G., Burke, D.J., and Sturgill, T.W. (2007). Golgi manganese transport is required for rapamycin signaling in *Saccharomyces cerevisiae*. Genetics **177,** 231-238.

Dunant, Y., Cordeiro, J.M., and Goncalves, P.P. (2009). Exocytosis, mediatophore, and vesicular Ca^{2+}/H^+ antiport in rapid neurotransmission. Ann N Y Acad Sci **1152,** 100-112.

Durr, G., Strayle, J., Plemper, R., Elbs, S., Klee, S.K., Catty, P., Wolf, D.H., and Rudolph, H.K. (1998). The medial-Golgi ion pump Pmr1 supplies the yeast secretory pathway with Ca^{2+} and Mn^{2+} required for glycosylation, sorting, and endoplasmic reticulum-associated protein degradation. Mol Biol Cell **9,** 1149-1162.

Ebert, A.M., Hume, G.L., Warren, K.S., Cook, N.P., Burns, C.G., Mohideen, M.A., Siegal, G., Yelon, D., Fishman, M.C., and Garrity, D.M. (2005). Calcium extrusion is critical for cardiac morphogenesis and rhythm in embryonic zebrafish hearts. Proc Natl Acad Sci U S A **102,** 17705-17710.

Edmond, C., Shigaki, T., Ewert, S., Nelson, M.D., Connorton, J.M., Chalova, V., Noordally, Z., and Pittman, J.K. (2009). Comparative analysis of CAX2-like cation transporters indicates functional and regulatory diversity. Biochem J **418,** 145-154.

Eide, D.J., Clark, S., Nair, T.M., Gehl, M., Gribskov, M., Guerinot, M.L., and Harper, J.F. (2005). Characterization of the yeast ionome: a genome-wide analysis of nutrient mineral and trace element homeostasis in *Saccharomyces cerevisiae*. Genome Biol **6,** R77.

Ettinger, W.F., Clear, A.M., Fanning, K.J., and Peck, M.L. (1999). Identification of a Ca^{2+}/H^{+} antiport in the plant chloroplast thylakoid membrane. Plant Physiol **119,** 1379-1386.

Evrard, Y.A., Mohammad-Zadeh, L., and Holton, B. (2004). Alterations in Ca^{2+}-dependent and cAMP-dependent signaling pathways affect neurogenesis and melanogenesis of quail neural crest cells in vitro. Dev Genes Evol **214,** 193-199.

Felle, H.H. (1994). The H^{+}/Cl- symporter in root-hair cells of *sinapis alba* (an electrophysiological study using ion-selective microelectrodes). Plant Physiol **106,** 1131-1136.

Felsenstein, J. (1997). An alternating least squares approach to inferring phylogenies from pairwise distances. Syst Biol **46,** 101-111.

Fischer, M., Schnell, N., Chattaway, J., Davies, P., Dixon, G., and Sanders, D. (1997). The *Saccharomyces cerevisiae* CCH1 gene is involved in calcium influx and mating. FEBS Lett **419,** 259-262.

Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B., Ljung, K., Sandberg, G., Hooykaas, P.J., Palme, K., and Offringa, R. (2004). A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science **306,** 862-865.

Gamborg, O.L., Murashige, T., Thorpe, T.A., and Vasil, I.K. (1976). Plant tissue culture media. In Vitro **12,** 473-478.

Ganesan, V. (2008). Rhizoremediation of cadmium soil using a cadmium-resistant plant growth-promoting rhizopseudomonad. Curr Microbiol **56,** 403-407.

Gehring, C.A., Irving, H.R., and Parish, R.W. (1990). Effects of auxin and abscisic acid on cytosolic calcium and pH in plant cells. Proc Natl Acad Sci U S A **87,** 9645-9649.

Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D., Jacq , C., Johnston , M., Louis, E.J., Mewes, H.W., Murakami, Y., Philippsen, P., Tettelin, H., and Oliver, S.G. (1996). Life with 6000 genes. Scinece **274,** 563-567.

Hagen, G., and Guilfoyle, T.J. (1985). Rapid induction of selective transcription by auxins. Mol Cell Biol **5,** 1197-1203.

Hagen, G., Uhrhammer, N., and Guilfoyle, T.J. (1988). Regulation of expression of an auxin-induced soybean sequence by cadmium. J Biol Chem **263,** 6442-6446.

Hirschi, K.D., Zhen, R.G., Cunningham, K.W., Rea, P.A., and Fink, G.R. (1996). CAX1, an H^+/Ca^{2+} antiporter from Arabidopsis. Proc Natl Acad Sci U S A **93,** 8782-8786.

Hirschi, K.D. (1999). Expression of Arabidopsis CAX1 in tobacco: altered calcium homeostasis and increased stress sensitivity. Plant Cell **11,** 2113-2122.

Hirschi, K.D., Korenkov, V.D., Wilganowski, N.L., and Wagner, G.J. (2000). Expression of arabidopsis CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. Plant Physiol **124,** 125-133.

Hirschi, K.D. (2003). Strike while the ionome is hot: making the most of plant genomic advances. Trends Biotechnol **21,** 520-521.

Hirschi, K.D. (2004). The calcium conundrum. Both versatile nutrient and specific signal. Plant Physiol **136,** 2438-2442.

Huang, X., and Saint-Jeannet, J.P. (2004). Induction of the neural crest and the opportunities of life on the edge. Dev Biol **275,** 1-11.

Hwang, I., Sze, H., and Harper, J.F. (2000). A calcium-dependent protein kinase can inhibit a calmodulin-stimulated Ca^{2+} pump (ACA2) located in the endoplasmic reticulum of *Arabidopsis*. Proc Natl Acad Sci U S A **97,** 6224-6229.

Iida, H., Nakamura, H., Ono, T., Okumura, M.S., and Anraku, Y. (1994). MID1, a novel *Saccharomyces cerevisiae* gene encoding a plasma membrane protein, is required for Ca^{2+} influx and mating. Mol Cell Biol **14,** 8259-8271.

Ivey, D.M., Guffanti, A.A., Zemsky, J., Pinner, E., Karpel, R., Padan, E., Schuldiner, S., and Krulwich, T.A. (1993). Cloning and characterization of a putative Ca^{2+}/H^{+} antiporter gene from *Escherichia coli* upon functional complementation of Na^{+}/H^{+} antiporter-deficient strains by the overexpressed gene. J Biol Chem **268,** 11296-11303.

Jin, S.W., Beis, D., Mitchell, T., Chen, J.N., and Stainier, D.Y. (2005). Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. Development **132,** 5199-5209.

Kamiya, T., and Maeshima, M. (2004). Residues in internal repeats of the rice cation/H^{+} exchanger are involved in the transport and selection of cations. J Biol Chem **279,** 812-819.

Kamiya, T., Akahori, T., and Maeshima, M. (2005). Expression profile of the genes for rice cation/H^{+} exchanger family and functional analysis in yeast. Plant Cell Physiol **46,** 1735-1740.

Kamiya, T., Akahori, T., Ashikari, M., and Maeshima, M. (2006). Expression of the vacuolar Ca^{2+}/H^{+} exchanger, OsCAX1a, in rice: cell and age specificity of expression, and enhancement by Ca^{2+}. Plant Cell Physiol **47,** 96-106.

Kasai, M., and Muto, S. (1990). Ca^{2+} pump and Ca^{2+}/H^{+} antiporter in plasma membrane vesicles isolated by aqueous two-phase partitioning from corn leaves. J Membr Biol **114,** 133-142.

Kellermayer, R., Aiello, D.P., Miseta, A., and Bedwell, D.M. (2003). Extracellular Ca^{2+} sensing contributes to excess Ca^{2+} accumulation and vacuolar fragmentation in a pmr1Delta mutant of S. cerevisiae. J Cell Sci **116,** 1637-1646.

Kerschen, A., Napoli, C.A., Jorgensen, R.A., and Muller, A.E. (2004). Effectiveness of RNA interference in transgenic plants. FEBS Lett **566,** 223-228.

Kingsbury, T.J., and Cunningham, K.W. (2000). A conserved family of calcineurin regulators. Genes Dev **14,** 1595-1604.

Korenkov, V., Hirschi, K., Crutchfield, J.D., and Wagner, G.J. (2007). Enhancing tonoplast Cd/H antiport activity increases Cd, Zn, and Mn tolerance, and impacts root/shoot Cd partitioning in *Nicotiana tabacum L.* Planta **226,** 1379-1387.

Koren'kov, V., Park, S., Cheng, N.H., Sreevidya, C., Lachmansingh, J., Morris, J., Hirschi, K., and Wagner, G.J. (2007). Enhanced Cd^{2+} -selective root-tonoplast-transport in tobaccos expressing *Arabidopsi*s cation exchangers. Planta **225,** 403-411.

Kreiling, J.A., Balantac, Z.L., Crawford, A.R., Ren, Y., Toure, J., Zchut, S., Kochilas, L., and Creton, R. (2008). Suppression of the endoplasmic reticulum calcium pump during zebrafish gastrulation affects left-right asymmetry of the heart and brain. Mech Dev **125,** 396-410.

Lacosta, A.M., Canudas, J., Gonzalez, C., Muniesa, P., Sarasa, M., and Dominguez, L. (2007). Pax7 identifies neural crest, chromatophore lineages and pigment stem cells during zebrafish development. Int J Dev Biol **51,** 327-331.

Lahner, B., Gong, J., Mahmoudian, M., Smith, E.L., Abid, K.B., Rogers, E.E., Guerinot, M.L., Harper, J.F., Ward, J.M., McIntyre, L., Schroeder, J.I., and Salt, D.E. (2003). Genomic scale profiling of nutrient and trace elements in *Arabidopsis thaliana.* Nat Biotechnol **21,** 1215-1221.

Lapinskas, P.J., Cunningham, K.W., Liu, X.F., Fink, G.R., and Culotta, V.C. (1995). Mutations in PMR1 suppress oxidative damage in yeast cells lacking superoxide dismutase. Mol Cell Biol **15,** 1382-1388.

Lasswell, J., Rogg, L.E., Nelson, D.C., Rongey, C., and Bartel, B. (2000). Cloning and characterization of IAR1, a gene required for auxin conjugate sensitivity in *Arabidopsis.* Plant Cell **12,** 2395-2408.

Li, W., and Cornell, R.A. (2007). Redundant activities of Tfap2a and Tfap2c are required for neural crest induction and development of other non-neural ectoderm derivatives in zebrafish embryos. Dev Biol **304,** 338-354.

Locke, E.G., Bonilla, M., Liang, L., Takita, Y., and Cunningham, K.W. (2000). A homolog of voltage-gated Ca^{2+} channels stimulated by depletion of secretory Ca^{2+} in yeast. Mol Cell Biol **20,** 6686-6694.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast **14,** 953-961.

Luo, G.Z., Wang, H.W., Huang, J., Tian, A.G., Wang, Y.J., Zhang, J.S., and Chen, S.Y. (2005). A putative plasma membrane cation/proton antiporter from soybean confers salt tolerance in *Arabidopsis*. Plant Mol Biol **59,** 809-820.

Luoni, L., Meneghelli, S., Bonza, M.C., and DeMichelis, M.I. (2004). Auto-inhibition of *Arabidopsis thaliana* plasma membrane Ca^{2+}-ATPase involves an interaction of the N-terminus with the small cytoplasmic loop. FEBS Lett **574,** 20-24.

Magidin, M., Pittman, J.K., Hirschi, K.D., and Bartel, B. (2003). ILR2, a novel gene regulating IAA conjugate sensitivity and metal transport in *Arabidopsis thaliana*. Plant J **35,** 523-534.

Marchi, V., Sorin, A., Wei, Y., and Rao, R. (1999). Induction of vacuolar Ca^{2+}-ATPase and H^+/Ca^{2+} exchange activity in yeast mutants lacking Pmr1, the Golgi Ca^{2+}-ATPase. FEBS Lett **454,** 181-186.

Matsumoto, T.K., Ellsmore, A.J., Cessna, S.G., Low, P.S., Pardo, J.M., Bressan, R.A., and Hasegawa, P.M. (2002). An osmotically induced cytosolic Ca^{2+} transient activates calcineurin signaling to mediate ion homeostasis and salt tolerance of *Saccharomyces cerevisiae*. J Biol Chem **277,** 33075-33080.

McAinsh, M.R., and Pittman, J.K. (2009). Shaping the calcium signature. New Phytol **181,** 275-294.

Mei, H., Zhao, J., Pittman, J.K., Lachmansingh, J., Park, S., and Hirschi, K.D. (2007). In planta regulation of the *Arabidopsis* Ca^{2+}/H^+ antiporter CAX1. J Exp Bot **58,** 3419-3427.

Miseta, A., Fu, L., Kellermayer, R., Buckley, J., and Bedwell, D.M. (1999). The Golgi apparatus plays a significant role in the maintenance of Ca^{2+} homeostasis in the vps33Delta vacuolar biogenesis mutant of *Saccharomyces cerevisiae*. J Biol Chem **274,** 5939-5947.

Miseta, A., Kellermayer, R., Aiello, D.P., Fu, L., and Bedwell, D.M. (1999). The vacuolar Ca^{2+}/H^+ exchanger Vcx1p/Hum1p tightly controls cytosolic Ca^{2+} levels in *S. cerevisiae*. FEBS Lett **451,** 132-136.

Morel, M., Crouzet, J., Gravot, A., Auroy, P., Leonhardt, N., Vavasseur, A., and Richaud, P. (2009). AtHMA3, a P1B-ATPase allowing Cd/Zn/Co/Pb vacuolar storage in *Arabidopsis*. Plant Physiol **149,** 894-904.

Muday, G.K., and Haworth, P. (1994). Tomato root growth, gravitropism, and lateral development: correlation with auxin transport. Plant Physiol Biochem **32,** 193-203.

Muller, E.M., Locke, E.G., and Cunningham, K.W. (2001). Differential regulation of two Ca^{2+} influx systems by pheromone signaling in *Saccharomyces cerevisiae.* Genetics **159,** 1527-1538.

Naseem, R., Holland, I.B., Jacq, A., Wann, K.T., and Campbell, A.K. (2008). pH and monovalent cations regulate cytosolic free Ca^{2+} in *E. coli*. Biochim Biophys Acta **1778,** 1415-1422.

Nasevicius, A., and Ekker, S.C. (2000). Effective targeted gene 'knockdown' in zebrafish. Nat Genet **26,** 216-220.

Nathan, D.F., Vos, M.H., and Lindquist, S. (1999). Identification of SSF1, CNS1, and HCH1 as multicopy suppressors of a *Saccharomyces cerevisiae* Hsp90 loss-of-function mutation. Proc Natl Acad Sci U S A **96,** 1409-1414.

Numata, M., and Orlowski, J. (2001). Molecular cloning and characterization of a novel $(Na^+,K^+)/H^+$ exchanger localized to the trans-Golgi network. J Biol Chem **276,** 17387-17394.

Ohyama, T., Igarashi, K., and Kobayashi, H. (1994). Physiological role of the chaA gene in sodium and calcium circulations at a high pH in *Escherichia coli*. J Bacteriol **176,** 4311-4315.

Olivero, I., Corbacho, I., and Hernandez, L.M. (2003). The ldb1 mutant of *Saccharomyces cerevisiae* is defective in Pmr1p, the yeast secretory pathway/Golgi Ca^{2+}/Mn^{2+}-ATPase. FEMS Microbiol Lett **219,** 137-142.

Ostergaard, S., Olsson, L., and Nielsen, J. (2000). Metabolic engineering of *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev **64,** 34-50.

Ouyang, M., Garnett, A.T., Han, T.M., Hama, K., Lee, A., Deng, Y., Lee, N., Liu, H.Y., Amacher, S.L., Farber, S.A., and Ho, S.Y. (2008). A web based resource characterizing the zebrafish developmental profile of over 16,000 transcripts. Gene Expr Patterns **8,** 171-180.

Page, R.D. (2002). Visualizing phylogenetic trees using TreeView. Curr Protoc Bioinformatics **Chapter 6,** Unit 6 2.

Paidhungat, M., and Garrett, S. (1997). A homolog of mammalian, voltage-gated calcium channels mediates yeast pheromone-stimulated Ca^{2+} uptake and exacerbates the cdc1(Ts) growth defect. Mol Cell Biol **17,** 6339-6347.

Palmer, C.P., Zhou, X.L., Lin, J., Loukin, S.H., Kung, C., and Saimi, Y. (2001). A TRP homolog in *Saccharomyces cerevisiae* forms an intracellular Ca^{2+}-permeable channel in the yeast vacuolar membrane. Proc Natl Acad Sci U S A **98,** 7801-7805.

Park, S., Cheng, N.H., Pittman, J.K., Yoo, K.S., Park, J., Smith, R.H., and Hirschi, K.D. (2005). Increased calcium levels and prolonged shelf life in tomatoes expressing *Arabidopsis* H^+/Ca^{2+} transporters. Plant Physiol **139,** 1194-1206.

Park, S., Kang, T.S., Kim, C.K., Han, J.S., Kim, S., Smith, R.H., Pike, L.M., and Hirschi, K.D. (2005). Genetic manipulation for enhancing calcium content in potato tuber. J Agric Food Chem **53,** 5598-5603.

Peiter, E., Fischer, M., Sidaway, K., Roberts, S.K., and Sanders, D. (2005). The *Saccharomyces cerevisiae* Ca^{2+} channel Cch1pMid1p is essential for tolerance to cold stress and iron toxicity. FEBS Lett **579,** 5697-5703.

Phillips, B.T., Bolding, K., and Riley, B.B. (2001). Zebrafish fgf3 and fgf8 encode redundant functions required for otic placode induction. Dev Biol **235,** 351-365.

Phillips, B.T., Kwon, H.J., Melton, C., Houghtaling, P., Fritz, A., and Riley, B.B. (2006). Zebrafish msxB, msxC and msxE function together to refine the neural-nonneural border and regulate cranial placodes and neural crest development. Dev Biol **294,** 376-390.

Pittman, J.K., and Hirschi, K.D. (2001). Regulation of CAX1, an *Arabidopsis* Ca^{2+}/H^+ antiporter. Identification of an N-terminal autoinhibitory domain. Plant Physiol **127,** 1020-1029.

Pittman, J.K., Shigaki, T., Cheng, N.H., and Hirschi, K.D. (2002). Mechanism of N-terminal autoinhibition in the *Arabidopsis* Ca^{2+}/H^+ antiporter CAX1. J Biol Chem **277,** 26452-26459.

Pittman, J.K., Sreevidya, C.S., Shigaki, T., Ueoka-Nakanishi, H., and Hirschi, K.D. (2002). Distinct N-terminal regulatory domains of Ca^{2+}/H^+ antiporters. Plant Physiol **130,** 1054-1062.

Pittman, J.K., Cheng, N.H., Shigaki, T., Kunta, M., and Hirschi, K.D. (2004). Functional dependence on calcineurin by variants of the *Saccharomyces cerevisiae* vacuolar Ca^{2+}/H^+ exchanger Vcx1p. Mol Microbiol **54,** 1104-1116.

Pittman, J.K., Shigaki, T., Marshall, J.L., Morris, J.L., Cheng, N.H., and Hirschi, K.D. (2004). Functional and regulatory analysis of the *Arabidopsis thaliana* CAX2 cation transporter. Plant Mol Biol **56,** 959-971.

Pozos, T.C., Sekler, I., and Cyert, M.S. (1996). The product of HUM1, a novel yeast gene, is required for vacuolar Ca^{2+}/H^+ exchange and is related to mammalian Na^+/Ca^{2+} exchangers. Mol Cell Biol **16,** 3730-3741.

Price, L.S., Langeslag, M., ten Klooster, J.P., Hordijk, P.L., Jalink, K., and Collard, J.G. (2003). Calcium signaling regulates translocation and activation of Rac. J Biol Chem **278,** 39413-39421.

Rajkumar, M., Nagendran, R., Lee, K.J., and Lee, W.H. (2005). Characterization of a novel Cr^{6+} reducing Pseudomonas sp. with plant growth-promoting potential. Curr Microbiol **50,** 266-271.

Rampey, R.A., Woodward, A.W., Hobbs, B.N., Tierney, M.P., Lahner, B., Salt, D.E., and Bartel, B. (2006). An *Arabidopsis* basic helix-loop-helix leucine zipper protein modulates metal homeostasis and auxin conjugate responsiveness. Genetics **174,** 1841-1857.

Randall, S.K., and Sze, H. (1986). Properties of the partially purified tonoplast H^+-pumping ATPase from oat roots. J Biol Chem **261,** 1364-1371.

Robert, H.S., and Offringa, R. (2008). Regulation of auxin transport polarity by AGC kinases. Curr Opin Plant Biol **11,** 495-502.

Robu, M.E., Larson, J.D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S.A., and Ekker, S.C. (2007). p53 activation by knockdown technologies. PLoS Genet **3,** e78.

Rudolph, H.K., Antebi, A., Fink, G.R., Buckley, C.M., Dorman, T.E., LeVitre, J., Davidow, L.S., Mao, J.I., and Moir, D.T. (1989). The yeast secretory pathway is perturbed by mutations in PMR1, a member of a Ca^{2+} ATPase family. Cell **58,** 133-145.

Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N., Weisbeek, P., and Scheres, B. (1999). An auxin-dependent distal organizer of pattern and polarity in the *Arabidopsis* root. Cell **99,** 463-472.

Sanders, D., Brownlee, C., and Harper, J.F. (1999). Communicating with calcium. Plant Cell **11,** 691-706.

Schallreuter, K.U., Kothari, S., Chavan, B., and Spencer, J.D. (2008). Regulation of melanogenesis--controversies and new concepts. Exp Dermatol **17,** 395-404.
Shigaki, T., and Hirschi, K. (2000). Characterization of CAX-like genes in plants: implications for functional diversity. Gene **257,** 291-298.

Shigaki, T., Cheng, N.H., Pittman, J.K., and Hirschi, K. (2001). Structural determinants of Ca^{2+} transport in the Arabidopsis H^+/Ca^{2+} antiporter CAX1. J Biol Chem **276,** 43152-43159.

Shigaki, T., Sreevidya, C., and Hirschi, K.D. (2002). Analysis of the Ca^{2+} domain in the *Arabidopsis* H^+/Ca^{2+} antiporters CAX1 and CAX3. Plant Mol Biol **50,** 475-483.

Shigaki, T., Pittman, J.K., and Hirschi, K.D. (2003). Manganese specificity determinants in the *Arabidopsis* metal/H^+ antiporter CAX2. J Biol Chem **278,** 6610-6617.

Shigaki, T., Barkla, B.J., Miranda-Vergara, M.C., Zhao, J., Pantoja, O., and Hirschi, K.D. (2005). Identification of a crucial histidine involved in metal transport activity in the *Arabidopsis* cation/H^+ exchanger CAX1. J Biol Chem **280,** 30136-30142.

Shigaki, T., Rees, I., Nakhleh, L., and Hirschi, K.D. (2006). Identification of three distinct phylogenetic groups of CAX cation/proton antiporters. J Mol Evol **63,** 815-825.

Shigaki, T., and Hirschi, K.D. (2006). Diverse functions and molecular properties emerging for CAX cation/H^+ exchangers in plants. Plant Biol (Stuttg) **8,** 419-429.

Shigeri, Y., Seal, R.P., and Shimamoto, K. (2004). Molecular pharmacology of glutamate transporters, EAATs and VGLUTs. Brain Res Brain Res Rev **45,** 250-265.

Solomon, K.S., Kudoh, T., Dawid, I.B., and Fritz, A. (2003). Zebrafish foxi1 mediates otic placode formation and jaw development. Development **130,** 929-940.

Sorin, A., Rosas, G., and Rao, R. (1997). PMR1, a Ca^{2+}-ATPase in yeast Golgi, has properties distinct from sarco/endoplasmic reticulum and plasma membrane calcium pumps. J Biol Chem **272,** 9895-9901.

Stathopoulos, A.M., and Cyert, M.S. (1997). Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast. Genes Dev **11,** 3432-3444.

Sze, H., Liang, F., Hwang, I., Curran, A.C., and Harper, J.F. (2000). Diversity and regulation of plant Ca^{2+} pumps: insights from expression in yeast. Annu Rev Plant Physiol Plant Mol Biol **51,** 433-462.

Tam, Y.Y., Epstein, E., and Normanly, J. (2000). Characterization of auxin conjugates in *Arabidopsis*. Low steady-state levels of indole-3-acetyl-aspartate, indole-3-acetyl-glutamate, and indole-3-acetyl-glucose. Plant Physiol **123,** 589-596.

Tissier, A.F., Marillonnet, S., Klimyuk, V., Patel, K., Torres, M.A., Murphy, G., and Jones, J.D. (1999). Multiple independent defective suppressor-mutator transposon insertions in *Arabidopsis*: a tool for functional genomics. Plant Cell **11,** 1841-1852.

Ton, V.K., and Rao, R. (2004). Functional expression of heterologous proteins in yeast: insights into Ca^{2+} signaling and Ca^{2+}-transporting ATPases. Am J Physiol Cell Physiol **287,** C580-589.

Trave, G., Pastore, A., Hyvonen, M., and Saraste, M. (1995). The C-terminal domain of alpha-spectrin is structurally related to calmodulin. Eur J Biochem **227,** 35-42.

Trave, G., Lacombe, P.J., Pfuhl, M., Saraste, M., and Pastore, A. (1995). Molecular mechanism of the calcium-induced conformational change in the spectrin EF-hands. EMBO J **14,** 4922-4931.

Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T.J. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell **9,** 1963-1971.

Venema, K., Belver, A., Marin-Manzano, M.C., Rodriguez-Rosales, M.P., and Donaire, J.P. (2003). A novel intracellular K^+/H^+ antiporter related to Na^+/H^+ antiporters is important for K^+ ion homeostasis in plants. J Biol Chem **278,** 22453-22459.

Wada, Y., Ohsumi, Y., Tanifuji, M., Kasai, M., and Anraku, Y. (1987). Vacuolar ion channel of the yeast, *Saccharomyces cerevisiae*. J Biol Chem **262,** 17260-17263.

Walz, A., Park, S., Slovin, J.P., Ludwig-Muller, J., Momonoki, Y.S., and Cohen, J.D. (2002). A gene encoding a protein modified by the phytohormone indoleacetic acid. Proc Natl Acad Sci U S A **99,** 1718-1723.

Webb, S.E., and Miller, A.L. (2006). Ca^{2+} signaling and early embryonic patterning during the blastula and gastrula periods of zebrafish and *Xenopus* development. Biochim Biophys Acta **1763,** 1192-1208.

Wehrle-Haller, B., Meller, M., and Weston, J.A. (2001). Analysis of melanocyte precursors in Nf1 mutants reveals that MGF/KIT signaling promotes directed cell migration independent of its function in cell survival. Dev Biol **232,** 471-483.

White, P.J., Pineros, M., Tester, M., and Ridout, M.S. (2000). Cation permeability and selectivity of a root plasma membrane calcium channel. J Membr Biol **174,** 71-83.

White, P.J., and Broadley, M.R. (2003). Calcium in plants. Ann Bot (Lond) **92,** 487-511.

Winslow, M.M., and Crabtree, G.R. (2005). Immunology. Decoding calcium signaling. Science **307,** 56-57.

Woodward, A.W., and Bartel, B. (2005). Auxin: regulation, action, and interaction. Ann Bot (Lond) **95,** 707-735.

Wu, G., Lewis, D.R., and Spalding, E.P. (2007). Mutations in *Arabidopsis* multidrug resistance-like ABC transporters separate the roles of acropetal and basipetal auxin transport in lateral root development. Plant Cell **19,** 1826-1837.

Yadav, J., Muend, S., Zhang, Y., and Rao, R. (2007). A phenomics approach in yeast links proton and calcium pump function in the Golgi. Mol Biol Cell **18,** 1480-1489.

Zhao, J., Barkla, B.J., Marshall, J., Pittman, J.K., and Hirschi, K.D. (2008). The *Arabidopsis* cax3 mutants display altered salt tolerance, pH sensitivity and reduced plasma membrane H^+-ATPase activity. Planta **227,** 659-669.

VITA

Name: Hui Mei

Address: 72 E 97th St. Apt 26, New York, New York 10029

Email: mhuicyto@gmail.com

Education: B.S. YunNan Universty, 1994

Ph.D., Molecular & Environmental Plant Science, Texas A&M University, 2009

Publications:

Mei H., Zhao J., Pittman J.K., Lachmansingh J., Park S., and Hirschi K.D. (2007) In planta regulation of the Arabidopsis Ca(2+)/H(+) antiporter CAX1. J Exp Bot. 2007 58(12):3419-3427.

Mei H., Shigaki T., and Hircshi K.D. Yeast as a tool for plant Ca^{2+} transport research. (in press).

Mei H., Cheng N.H., Pittman J.K., Park S., Escareno R.A., and Hirschi K.D. Root development under metal stress in *Arabidopsis thaliana* requires the H^+/cation antiporter CAX4 (in press).

Mei H., Sweet E.M., Shigaki T., Riley B.B., and Hirschi K.D. An endomembrane Ca^{2+}/H^+ antiporter is required for zebrafish (*Danio rerio*) neural crest development. (in submission)

Zhao J., Shigaki T., Mei H., Guo Y., Cheng N.H., and Hirschi K.D. (2009) Interaction between *Arabidopsis* Ca^{2+}/H^+ exchangers CAX1 and CAX3. J Biol Chem. 248(7):4605-4615.

Shigaki T.,Mei H.,Marshal J., and Hirschi K.D. Removal of 3'-UTR from the cDNA of *Arabidopsis* cation/H^+ exchanger CAX1 activates Ca^{2+} and Mn^{2+} transport in yeast (in preparation).

Shigaki T.,Mei H.,Marshal J., and Hirschi K.D. Deletion of entire N-terminal tail is required for the activation of *Arabidopsis* Ca^{2+}/H^+ exchanger CAX3 (in preparation).